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Pleurotus ostreatus photo by Steve Balcombe

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Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

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Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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Nutritional value and safety of castor bean (*Ricinus communis*) seeds detoxified in solid-state fermentation by *Pleurotus ostreatus*

OLUWAFOLAKEMI CHRISTIANAH ADEBAYO[1], CLEMENT OLUSOLA OGIDI[1, 2♥], BAMIDELE JULIET AKINYELE[1]

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Abstract. *Adebayo OC, Ogidi CO, Akinyele BJ. 2019. Nutritional value and safety of castor bean (Ricinus communis) seed detoxified in solid-state fermentation by Pleurotus ostreatus. Biofarmasi J Nat Prod Biochem 17: 55-64.* The nutrient and antinutrient contents of unfermented castor seeds (UCS), castor seeds fermented with *Pleurotus ostreatus* (CPF), and "Ogiri," a naturally fermented condiment from castor seeds (CSF), were determined. Proximate analyses of all the samples were carried out using methods of the Association of Official Agricultural Chemists. The safety of the samples was carried out using an animal model. The raw castor seeds (UCS) had the highest carbohydrates (61.04%), ash (6.02%), fat (6.65%), fiber (6.62%), and calcium (0.30 mg/100g). Castor seeds fermented with *Pleurotus ostreatus* (CPF) had the highest protein content of 20.47%, magnesium of 7.16 mg/100g, alkaloids (7.40 mg/g), and saponins (6.69 mg/g). CSF had the highest zinc (0.69 mg/100g). CPF had the lowest tannin (0.05 mg/g). The essential amino acids increased significantly ($p<0.05$) in the fermented samples. Tryptophan was absent in UCS but present in CSF and CPF with values of 0.78 mg/100g and 1.15 mg/100g, respectively. The hematological analysis of the rats fed CPF had the highest WBC of 5.43×10^9, which indicated a positive immunomodulatory effect. Hence, this study revealed that *Pleurotus ostreatus* degraded the toxic compounds in castor seeds to a large extent and enhanced the nutritional contents of the final product.

Keywords: Castor seeds, fermentation, hematological analysis, nutrients, *Pleurotus ostreatus*

INTRODUCTION

Natural toxins produced by plants are secondary metabolites that protect them from various threats from microorganisms, insects, and predators (Singh et al., 2013). Many plants contain natural toxins, such as bitter apricot seeds, bamboo shoots, cassava, flaxseeds, potatoes, wild mushroom, green beans, red and white kidney beans, and castor seeds (RAS 2007). These toxins, namely; cyanogenic glycosides, glycoalkaloids, muscarine, and lectins, may be found throughout the whole plant or in some parts of the plants (seeds, fruits, and leaves). These toxins could be harmful to human health when ingested in a significant amount or when raw materials are not processed properly into final food products.

The Castor oil plant (*Ricinus* communis) belongs to the family of *Euphorbiaceae* and grows throughout tropical and sub-tropical regions of the world (Ojinnaka et al., 2013; Sousa et al., 2017). Castor seed is a poisonous seed of the castor oil plant (*R. communis).* The castor seed is inedible because the seed contains a toxic protein, ricin, and other toxic constituents such as ricinine and ricinoleic acids (Madeira Jr. et al., 2011). The presence of toxic components in castor seeds has remained a serious impediment to the consumption of castor seeds. Various detoxification methods have been used with varying degrees of success and limitations (Akande et al., 2016). From an economic point of view, these processes are still not practical and not efficient enough to be applied on a large scale.

Fermentation is one of the oldest forms of biotechnology that uses microorganisms to produce secondary metabolites and recombinant products on an industrial scale (Paulová et al., 2016). The fermentation processes can also enhance food safety by reducing toxic compounds during the production process. Some examples of traditionally fermented condiments produced by solid-substrate fermentation are "Iru" from soya beans (*Glycine max*), Bambara nut (*Vigna subterrenea*), African locust bean (*Parkia biglobosa),* "Okpehe" from African Mesquite seeds (*Prosopis Africana),* "Ugba" from oil bean seed (*Pentaclethra macrophylla*) and "Ogiri*"* from melon seeds (*Cucumeropsis mannii*) or castor seeds (*Ricinus communis*)

In Nigeria, 'Ogiri,' a condiment from fermented castor seeds, has a characteristic ammoniacal odor, and its flavor enhances the taste of traditional soups and sauces (Omafuvbe et al. 2004). It serves as a supplement, a nutritious non-meat protein substitute, and a functional ingredient in soup. Although most of the condiments constitute a significant nutritional proportion of diet for many people, there are still a lot of challenges associated with their products, such as long hours of boiling, recurrent boiling before the fermentation, having a short shelf life, less acceptable packaging materials, and the typical putrid odor (Ishiwu et al. 2015). Therefore, a new method needs to be introduced by utilizing microorganisms (fungi) to make it easier to reduce toxins in the substrate. The use of

fungi for eliminating toxins has been adopted in several traditional foods to facilitate the production process.

Some fermented foods have been known in several countries. Aidoo et al. (2006) used *Aspergillus oryzae* for the fermentation process of rice before introducing other yeast to produce Sake (rice wine). 'Tempeh,' traditional food from Indonesia, was produced by fermenting cooked soybeans with a fast-growing fungus: *Rhizopus oligosporus* (Iljas et al. 1973). Soy sauce, a condiment made from wheat and soybeans, was fermented with *Aspergillus* spp., yeasts, and lactic acid bacteria. 'Furu,' a condiment side dish consisting of soya bean curd fermented and partly degraded by the mold; *Actinomucor elegans* (Nout and Aidoo 2011). Fungi such as *P. ostreatus* possess several lignocellulolytic enzymes (laccase, manganese peroxidase, cellulase, and xylanase), selectively used to degrade the toxic compounds in some crops (da Luz et al. 2014). Since *P. ostreatus* can be tagged as a Generally recognized as safe (GRAS) fungus, it is a non-pathogenic microorganism. It is commonly used to degrade and detoxify food crops before consumption. Therefore, this study was designed to detoxify castor seeds using *P. ostreatus* in solid-state fermentation and enhance the by-product's nutritional properties.

MATERIALS AND METHODS

Collection of castor seeds

Castor seeds were obtained from Akure main market. The raw castor seeds were dehulled, sorted out to remove unwanted materials, and dried at 27ºC for 7 days. 'Ogiri,' a locally fermented condiment from castor seeds (CSF), was purchased.

Fungal cultivation

The spawn of *Pleurotus ostreatus* was obtained from the Federal Institute of Industrial Research, Oshodi (FIIRO), Lagos. The spawn was inoculated into Potato Dextrose Agar (PDA) plate and incubated for seven days at 25ºC.

Fermentation of castor seeds with *Pleurotus ostreatus*

The dehulled castor seeds were divided into two portions. The first portion was boiled for 1 hour, while the second portion was not boiled and left unfermented (UCS). Four agar discs (6 mm) of *Pleurotus ostreatus* mycelia were inoculated into sterile Potato dextrose broth (20 ml) and left for seven days at 25ºC. After that, the broth was aseptically introduced to boiled castor seeds (500 g) and left to ferment for five days using solid-state fermentation. After fermentation, the castor seeds (CPF) were mashed into paste and oven-dried at 28ºC for 4 hours.

Proximate analysis of unfermented and fermented castor seeds

Proximate analysis of raw and fermented castor seeds was determined using the method of the Association of Official Analytical Chemists (AOAC) (2016).

The sample's moisture was determined by drying 2 g of the sample in the oven at 105ºC for 3 hours.

$$\% \text{ Moisture content} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where, W_1=Weight of crucible; W_2=Weight of crucible + sample before drying; W_3=Weight of crucible + sample after drying

The ash content of the sample was determined by incinerating 5.0 g of sample in a muffle furnace at 550ºC for 24 h.

$$\% \text{ Total ash} = \frac{W_5 - W_6}{W_4} \times 100$$

Where, W_4=Weight of sample before ashing (g); W_5= Weight of dish + sample after ashing; W_6=Weight of empty dish

The fat content was determined by extracting fat from samples using n-hexane in a Soxhlet extractor. Briefly, 2 g of sample was wrapped in a filter paper and gradually lowered in the thimble with a fitted flask containing n-hexane. The round-bottomed flask in a Soxhlet extraction unit was slowly heated with a thermostatically controlled mantle. The solvent evaporated and passed through the sides tube of the extract to the reflux condenser. The filter paper with the defatted sample was removed from the extractor, and the refluxed solvent was distilled out and recovered.

$$\% \text{ Crude fat} = \frac{W_2 - W_1}{S} \times 100$$

Where, W1=Weight of empty evaporating dish; W2= Weight of evaporating dish + content after drying; S=Weight of the sample after drying.

The protein content was determined using the Kjeldahl nitrogen method. The sample (2.0g) was weighed into the digesting flask. Kjeldahl catalyst and 20 ml of concentrated H_2SO_4 were added to the sample and then fixed in the digestion unit (450 ºC) of the Kjeldahl apparatus in a fume cupboard for 8 hours. After reaching room temperature, boric acid (20 ml of 4%) was pipetted into a conical flask. A drop of methyl red was added to the flask as an indicator. The sample was diluted with 75 ml of distilled water made alkaline with 20 ml of NaOH (20%) and distilled. The steam exit of the distillatory was closed, and the change in color of boric acid to green was timed. The mixture was distilled for 15 min. The filtrate was then titrated against 0.1N HCl. The equation calculated the protein content (% of Protein) :

$$\% \text{ Protein content} = \text{nitrogen content} \times 6.25.$$

Fiber content was determined using acid and alkaline digestion methods with 20% H_2SO_4 and NaOH solution.

$$\% \text{ Fiber content} = \frac{W_2 - W_3}{W_1} \times 100$$

Carbohydrate content was determined by the difference method with the equation as follows:

Carbohydrates (%) = 100-(% moisture +% protein +% fat +% crude fiber +% ash)

Mineral analysis of unfermented and fermented castor seeds

AOAC (2013) method was used to determine the mineral composition of the unfermented and fermented castor seeds. Two g of sample was weighed into a crucible and heated in a muffle furnace at 550ºC for 6 hours. The ash was cooled, and 6N HCl was added. The mixture was boiled for 10 min, cooled, and filtered into a 100 ml volumetric flask. The crucible was washed with distilled water, added the filtrate was put into a 100 ml volumetric flask. The volume was made to 100 ml with distilled water. An aliquot of the filtrate was aspirated into the Atomic Absorption Spectrophotometer (Pye Unicam). The absorbance values corresponding to the different minerals were recorded against standard solutions of Ca, Mg, Mn, Fe, Cu, Zn, and Pb.

Phytochemicals analysis of unfermented and fermented castor seeds

The qualitative and quantitative analysis of alkaloids, tannins, saponins, and flavonoids were determined by using the methods of Trease and Evans (2005), Sofowora (1993), Harborne and Baxter (1995), and Trease and Evans (2005). Briefly, for alkaloids, the sample was stirred with 5 ml of 10% aqueous hydrochloric acid in a hot water bath, and a few drops of Dragendoff's reagent was added. The occurrence of orange-red crystalline precipitate indicated the presence of alkaloids. Saponin, 5 g of the sample was grounded, and 10 ml of 20% aqueous ethanol was added. The mixture was stirred continuously for 4 hours in a hot water bath at about 55ºC. The mixture was filtered, and the residue was re-extracted using 20% ethanol. The concentrate was transferred into a 250 mL separating funnel, and 20 ml of diethyl ether was added and shaken together. The aqueous layer was recovered, while the ether was later discarded. The purification process was repeated. 60 ml of n-butanol was added and washed twice with 10 ml of 5% aqueous NaCl (Obadoni and Ochuko 2001). Saponin content was calculated as:

$$\%\text{Saponin} = \frac{\text{Initial weight} - \text{final weight of the sample}}{\text{Initial weight}} \times 100$$

Flavonoid was quantified by boiling 5 g of each processed sample in 100 ml of 2 M HCl solution for 40 mins. It was allowed to cool to room temperature before filtered through Whatman filter paper. A flavonoid in the filtrate was precipitated by drop-wise addition of concentrated ethyl acetate until in excess. Following filtration, the flavonoid precipitate recovered was oven-dried, and the weight of flavonoid was obtained by difference and expressed as a percentage of the sample analyzed (Obadoni and Ochuko 2001).

Tannin was determined by adding 40 ml diethyl ether containing 1% acetic acid (v/v) to 400 mg of each sample. The mixtures were mixed to remove pigment materials. The supernatant was carefully discarded after 5 mins, and 20 ml of 70% aqueous acetone was added. The flask was sealed with a cotton plug covered with aluminum foil, then kept in a shaker for 2 hours for extraction. The flask content was filtered through the Whatman filter paper (No. 2). Aliquot 0.5 ml filtrate was made up to 1.0 ml with distilled water and 0.5 ml Folin Ciocalteu reagent added and then mixed properly before 2.5 ml of 20% sodium carbonate solution was added and further mixed. The mixtures were kept for 40 mins at room temperature, after which absorbance was taken using a spectrophotometer, and concentration was estimated from the tannic acid standard curve (Sarkiyayi and Agar 2010).

The concentration of phenolics in the sample was determined using the spectrophotometric method at λmax = 765 nm (Singleton et al. 1999). The reaction mixture was prepared by mixing 0.5 ml of a methanolic solution of the sample, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 ml of 7.5% $NaHCO_3$. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 ml of 7.5% of $NaHCO_3$. After that, the samples were ŧ incubated at 45ºC for 45 min. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid, and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolics was determined (mg/ml) from the calibration curve. The phenolics content in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Determination of amino acids in unfermented and fermented castor seeds

Initially, 200 mg of each ground seed sample was defatted with a chloroform/methanol mixture in a ratio of 1: 1. Then, 30 mg of the defatted sample was put into a glass ampoule, added 7 ml of 6 M HCl, and oxygen was expelled by passing nitrogen into the ampoule. The sealed ampoule was put in the oven at 105ºC for 22 hours, allowed to cool, and filtered. The filtrate was then evaporated to dryness at 40ºC under vacuum in a rotary evaporator. The residue was dissolved with 5 ml acetate buffer (pH 2.0) and loaded into the amino acid analyzer, where the amino acid compositions of the seed samples were determined by the Ion Exchange Chromatographic (IEC) method using the Technicon Sequential Multisample Amino Acid Analyzer (Technicon Instruments Corporation, New York) (Spackman et al. 1958).

Experimental design for *in vivo* animal model

Twenty albino rats were procured from the Department of Animal Production and Health Science, Federal University of Technology, Akure, Nigeria. The rats were weighed and divided into four groups. They were fed on a basal diet and water for seven days before being fed a different diet. The rats were grouped as follows: (i) BD: Animal fed basal diet only, (ii) BD+UCS: Animal fed basal diet and ground unfermented castor seed, (iii) BD+CSF: Animal fed basal diet and commercial fermented castor seeds ("Ogiri"), (iv) BD+CPF: Animal fed basal diet and castor seed fermented with *Pleurotus ostreatus*. The

feeding trials were carried out for ten days before the rats were sacrificed.

Collection of blood sample

Blood samples were collected by direct cardiac puncture and dispensed into EDTA bottles. The blood samples collected were tested for the red blood cell (RBC) counts, White Blood Cell (WBC) counts, hemoglobin (Hb) concentration, and Packed Cell Volume (PCV) according to the method described by Chesbrough (2000).

Histopathological analysis

Histopathological examination was carried out according to Sarkar et al. (2005). Briefly, the liver and kidneys were dissected and fixed instantaneously in 10% formal saline for 24 hours. The specimens were washed under tap water, dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin wax (melting point of 50-56 ^{o}C). Paraffin sections were cut at six µm thicknesses using a rotary microtome; the sections were stained with Harris hematoxylin and eosin. An automatic photomicrographic system made the observation using a light microscope, and photographs were taken.

Statistical analysis

Data obtained from the study were subjected to analysis of variance (ANOVA) using statistical software, SPSS version 22.0, and judged significantly at a 95% confidence level ($P<0.05$).

RESULTS AND DISCUSSION

Proximate and mineral composition of the unfermented and fermented castor seeds

Castor seeds fermented with *Pleurotus ostreatus* (CPF) had the highest protein content of 20.47%, while CSF had the highest moisture content of 17.29%. UCS had the highest ash (6.02%), fats (6.65%), and fiber (6.62%) (Figure 1). Table 1 shows the mineral composition of raw and fermented castor seeds. CPF had the highest magnesium composition of 7.16 mg/g. CSF had the highest zinc of 0.69 mg/g. UCS had the highest calcium of 0.30 mg/g.

Phytochemical constituents of the unfermented and fermented castor seeds

There was no significant difference ($p<0.05$) in the tannin content of CSF (0.06 mg/g) and CPF (0.05 mg/g). There was a significant increase ($p<0.05$) in the alkaloid (7.40 mg/g) and saponin contents (6.69 mg/g) of CPF. There was a slightly significant difference in the phenol contents of the unfermented and fermented samples: UCS (1.30 mg/g), CSF, and CPF (1.20 mg/g). There were significant differences in flavonoid contents of the samples: UCS (0.65 mg/g), CSF (0.60 mg/g) and CPF (0.48 mg/g).

Amino acid concentration of the unfermented and fermented castor seeds

Table 3 revealed the amino acids in the unfermented (UCS) and fermented castor seeds (CSF and CPF). Tryptophan was absent in UCS but had values of 0.78 mg/g and 1.15 mg/g in CSF and CPF, respectively. Glutamic acid had the highest concentration in all the samples: UCS had a composition of 10.02 mg/g, CSF had a composition of 10.22 mg/g, and CPF had a composition of 14.59 mg/g. Glutamine and asparagine were absent in all the samples.

Hematological studies of the albino rats were used to test for the safety of the unfermented and fermented castor seeds

Hematological analysis of the rats was tested for safety for unfermented and fermented castor seeds. Rats fed commercial fermented castor seeds (BD+CSF) had the highest Packed Cell Volume (PCV) of 30.67%, while rats fed raw castor seeds (BD+UCS) had the lowest PCV of 16.33%. Rats fed castor seeds fermented with *Pleurotus ostreatus* (BD+CPF) had the highest White Blood Cell (WBC) of 5.43 g/L, while rats fed raw castor seeds (BD+UCS) had the lowest WBC of 3.33 g/L. Rats fed basal diet (BD) had the highest Red Blood Cell (RBC) of 3.42 g/L, while rats fed raw castor seeds (BD+UCS) had the lowest RBC of 1.66 g/L. Rats fed basal diet (BD) had the highest hemoglobin levels of 10.47 g/L, while rats fed raw castor seeds (BD+UCS) had the lowest hemoglobin level of 4.47 g/L. Table 5 shows the differential White Blood Cell Count of rats tested for biosafety. BD+ UCS group had the highest lymphocyte count of 59.33%, while the BD group had the lowest lymphocyte count, highest neutrophil (46.67%), eosinophil (2.00%), and monocyte (2.00%) count. BD+CPF group had no count for neutrophils, eosinophils, and monocyte. All the groups had no basophil count.

Histopathological studies of the albino rats were used to test for the safety of the unfermented and fermented castor seeds

Figure 2 shows the photomicrograph of the kidneys of rats used to test for biosafety of the raw and fermented castor seed. Kidney of rats fed basal diet (BD) and kidneys of rats fed commercial fermented castor seed (BD+CSF) had the normal histological structure showing the glomerulus, Bowman's capsule, and lymphatic vessels (LV). The kidney of rats fed raw castor seed (BD+UCS) showed enlargement of Bowman's capsule (EBC), shrunken glomerulus (SG), and distorted proximal and distal convoluted tubules (Z). The kidneys of rats fed castor seed fermented with *Pleurotus ostreatus* (BD+CPF) showed disruption of glomerular tuft (D) and hemorrhagic lesions (I). Figure 3 shows the photomicrograph of livers of rats fed raw and fermented castor seeds. Liver of rats fed basal diet and liver of rats fed fermented castor seed showed normal liver structure of sinusoid cells (O) and hepatocytes (N). The liver of rats fed raw castor seed showed dilation of the sinusoid cells (T) and vascular congestion (K). The liver of rats fed castor seeds fermented with *Pleurotus ostreatus* showed dilation of the sinusoid cells (T), vascular congestion (K), and degenerated hepatocytes (DH).

Table 1. Mineral composition (mg/g) of unfermented and fermented castor seeds

Minerals	UCS	CPF	CSF
Lead	0.03±0.00^{b}	0.02±0.00^{a}	0.02±0.00^{a}
Zinc	0.13±0.00^{a}	0.38±0.00^{b}	0.69±0.00^{c}
Iron	0.17±0.00^{a}	0.75±0.00^{c}	0.36±0.00^{b}
Nickel	0.03±0.00^{c}	0.01±0.00^{b}	0.00±0.00^{a}
Chromium	0.01±0.01^{b}	0.00±0.00^{a}	0.00±0.00^{a}
Magnesium	3.79±0.01^{a}	7.16±0.00^{c}	6.86±0.01^{b}
Manganese	0.04±0.00^{a}	0.21±0.00^{c}	0.21±0.00^{c}
Copper	0.10±0.00^{a}	0.21±0.00^{c}	0.19±0.00^{b}
Calcium	0.30±0.00^{b}	0.10±0.00^{a}	0.10±0.00^{a}

Note: Values carrying the same alphabet in the same row are not significantly different (p>0.05). Values are means of triplicates ±SD. UCS: Unfermented castor seeds, CPF: Castor seeds fermented with *Pleurotus ostreatus*, CSF: Commercial fermented castor seeds

Table 2. Quantitative phytochemical compositions (mg/g) of unfermented and fermented castor seeds

Parameters	UCS	CPF	CSF
*Phenol	1.30±0.01^{b}	1.20±0.00^{a}	1.20±0.01^{a}
Tannin	0.10±0.01^{b}	0.05±0.00^{a}	0.06±0.01^{a}
Alkaloids	0.99±0.01^{a}	7.40±0.01^{c}	1.90±0.01^{b}
Flavonoids	0.65±0.01^{c}	0.48±0.01^{a}	0.60±0.01^{b}
Saponins	1.95±0.01^{b}	6.69±0.01^{c}	1.40±0.01^{a}

Note: Values carrying the same alphabet in the same row are not significantly different (p>0.05). Values are means of triplicates ±SD. UCS: Unfermented castor seeds CPF: Castor seeds fermented with *Pleurotus ostreatus* CSF: Commercial Fermented castor seed, *expressed in terms of Gallic acid equivalent (mg of GA/g of extract)

Table 3. Amino acid composition (mg/g) of unfermented and fermented castor seeds

Amino acids	UCS	CSF	CPF
Leucine	6.55±0.26^{a}	7.58±0.02^{b}	9.92±0.02^{c}
Lysine	4.49±0.10^{b}	3.71±0.02^{a}	5.62±0.02^{c}
Phenylalanine	2.60±0.15^{a}	3.97±0.02^{b}	4.61±0.02^{c}
Isoleucine	1.64±0.20^{a}	3.81±0.01^{b}	4.30±0.02^{c}
Tryptophan	0.00±0.00^{a}	0.78±0.03^{b}	1.15±0.01^{c}
Valine	2.73±0.20^{a}	3.97±0.01^{b}	3.97±0.02^{b}
Methionine	0.62±0.03^{a}	0.87±0.01^{b}	2.03±0.03^{c}
Proline	2.96±0.10^{b}	2.35±0.02^{a}	4.06±0.02^{c}
Arginine	4.67±0.18^{a}	5.31±0.02^{a}	8.52±0.03^{b}
Tyrosine	1.85±0.05^{a}	2.77±0.02^{b}	4.32±0.02^{c}
Histidine	1.57±0.11^{a}	2.36±0.02^{c}	2.22±0.02^{b}
Cystine	0.73±0.08^{a}	0.97±0.02^{b}	1.20±0.01^{c}
Alanine	2.58±0.35^{a}	3.87±0.02^{b}	4.32±0.02^{c}
Glutamic acid	10.00±0.12^{a}	10.22±0.02^{b}	14.59±0.02^{c}
Glycine	1.26±0.29^{a}	3.52±0.02^{b}	3.95±0.01^{c}
Threonine	1.56±0.01^{a}	3.02±0.02^{b}	4.15±0.01^{c}
Serine	3.32±0.38^{b}	2.32±0.00^{a}	3.62±0.00^{b}
Aspartic acid	7.69±0.15^{a}	7.81±0.03^{a}	12.59±0.01^{b}
Glutamine	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Asparagine	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Note: Values carrying the same superscript in the same row are not significantly different (p>0.05). Values are means of triplicates ±SD UCS: Unfermented castor seeds CPF: Castor seeds fermented with *Pleurotus ostreatus* CSF: Commercial fermented castor seeds.

Table 4. Hematological parameters of rats fed unfermented and fermented castor seeds

Groups	PCV (%)	Hb (g/L)	WBC (10^{9} g/L)	RBC (10^{12}g/L)
BD+UCS	16.33±1.53^{a}	4.47±0.31^{a}	3.33±0.21^{a}	1.66±0.03^{a}
BD+CSF	30.67±1.15^{c}	8.43±0.35^{c}	4.30±0.30^{b}	2.23±0.15^{b}
BD+CPF	22.00±0.00^{b}	7.47±0.15^{b}	5.43±0.31^{c}	2.49±0.10^{c}
BD	31.67±1.53^{c}	10.47±1.15^{d}	4.17±0.15^{b}	3.42±0.66^{d}

Note: Values carrying the same alphabet in the same column are not significantly different (p>0.05). Values are means of triplicates ±SD BD: Fed basal diet only, BD+UCS: Fed basal diet mixed with ground unfermented castor seeds, BD+CSF: Fed basal diet mixed with commercial fermented castor seeds, BD+CPF: Fed basal diet mixed with castor seed fermented with *Pleurotus ostreatus.*

Table 5. Differential white blood cell count of rats fed unfermented and fermented castor seeds

Group	Lymphocytes (%)	Neutrophil (%)	Eosinophil (%)	Monocytes (%)	Basophil (%)
BD+UCS	59.33±1.15^{c}	38.00±2.00^{b}	1.00±0.00^{b}	1.00±0.00^{b}	0.00±0.00
BD+CSF	48.00±0.00^{b}	41.33±1.15^{c}	1.67±0.58^{c}	1.67±0.58^{c}	0.00±0.00
BD+CPF	50.00±2.00^{b}	31.33±1.53^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00
BD	46.00±2.00^{a}	46.67±1.15^{d}	2.00±0.00^{c}	2.00±0.00^{c}	0.00±0.00

Note: Values carrying the same alphabet in the same column are not significantly different (p>0.05). Values are means of triplicates ±SD. BD: Fed basal diet only, BD+UCS: Fed basal diet mixed with ground unfermented castor seeds, BD+CSF: Fed basal diet mixed with commercial fermented castor seeds, BD+CPF: Fed basal diet mixed with castor seed fermented with *Pleurotus ostreatus*

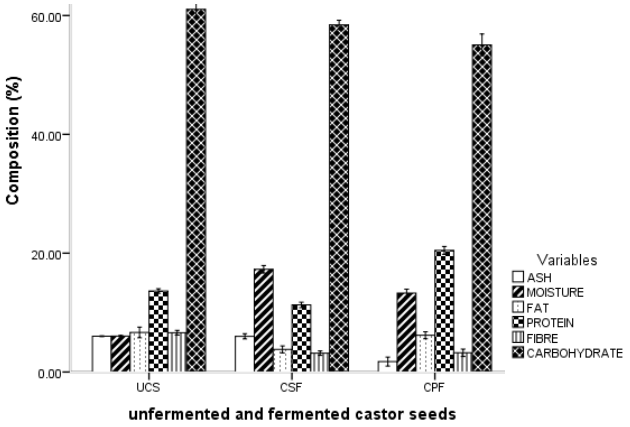

Figure 1. Proximate composition of unfermented and fermented castor seeds. Note: UCS: Unfermented castor seeds, CSF: Fermented castor seeds, CPF: Castor seeds fermented with *Pleurotus ostreatus*

Discussion

The proximate analysis revealed that the fermented castor seeds had higher moisture content than the unfermented ones. The increase in the moisture content could be attributed to the moisture absorbed by the seeds during boiling before fermentation. This result agrees with Ishiwu et al. (2015) that the moisture content of the fermented castor seeds increased after fermentation. However, there was a reduction in the moisture content of castor seeds fermented with *Pleurotus ostreatus* (CPF) compared to 'Ogiri,' the naturally fermented condiment from castor seeds (CSF). This result agreed with Ajala and Akinterinwa's (2016) findings which evaluated the nutrient quality of dried 'Ogiri' produced from melon seeds. The researchers observed a reduction in the moisture content.

CPF had the highest protein content among the three samples (20.47%), while CSF had the lowest (11.14%). This result is in accordance with the findings of Bao et al. (2013), who observed an increase in the protein content (28.17% to 133.42%) of rice fermented with *Pleurotus eryngii*. The increase in the protein content could be due to leaching out of some soluble substances present in the seeds (tannin, trypsin inhibitor) that are protein-bound (Kpanja et al. 2016). The increase in the protein contents could also be due to the bioconversion of crude fiber and carbohydrates in the unfermented castor seeds into mycelia protein or single-cell protein (Alemawor et al., 2009). Deepalakshmi and Mirunalini (2014) reported that *Pleurotus ostreatus* are rich in diverse proteins, which possibly enhance the protein contents of CPF, thereby making its protein content higher than others. The findings of Espinosa-Páez et al. (2017) enhanced the protein digestibility of *Avena sativa* and *Phaseolus vulgaris* by fermentation with *Pleurotus ostreatus.*

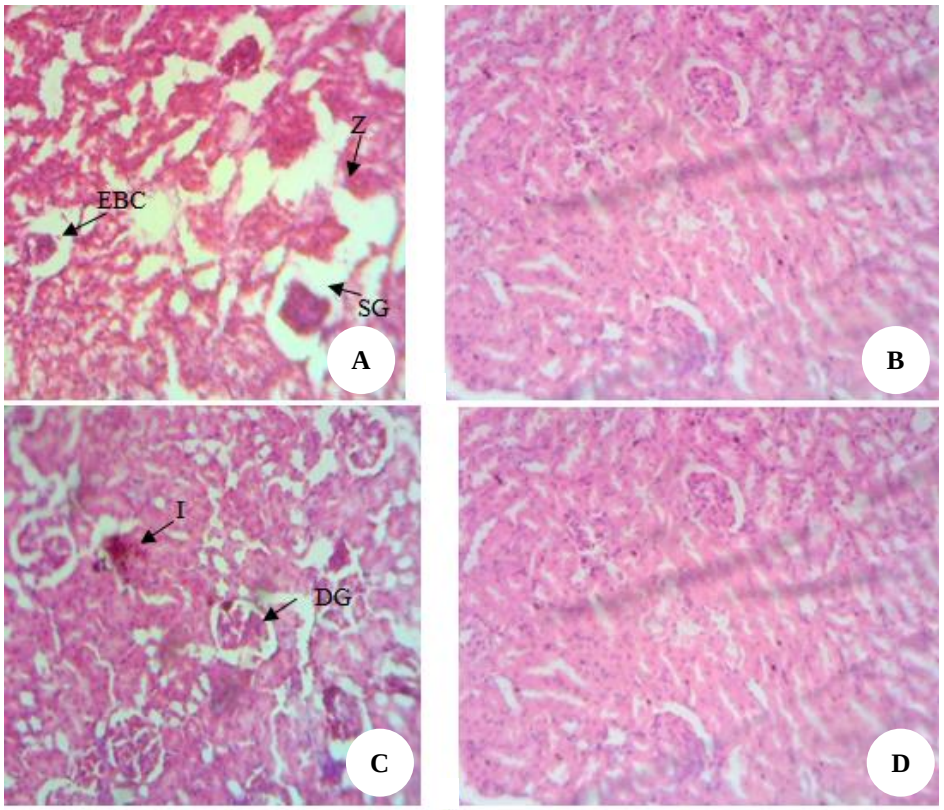

Figure 2. Photomicrograph of kidneys of rats fed unfermented and fermented castor seeds. A: fed raw castor seeds (BD+UCS), B: fed commercial fermented castor seeds (BD+CSF), C: fed castor seeds fermented with *Pleurotus ostreatus* (BD+CPF), D: fed basal diet (BD). Enlargement of Bowman's capsule (EBC), shrunken glomerulus (SG) and distorted proximal and distal convoluted tubules (Z), disruption of glomerular tuft (D), and hemorrhagic lesions (I).

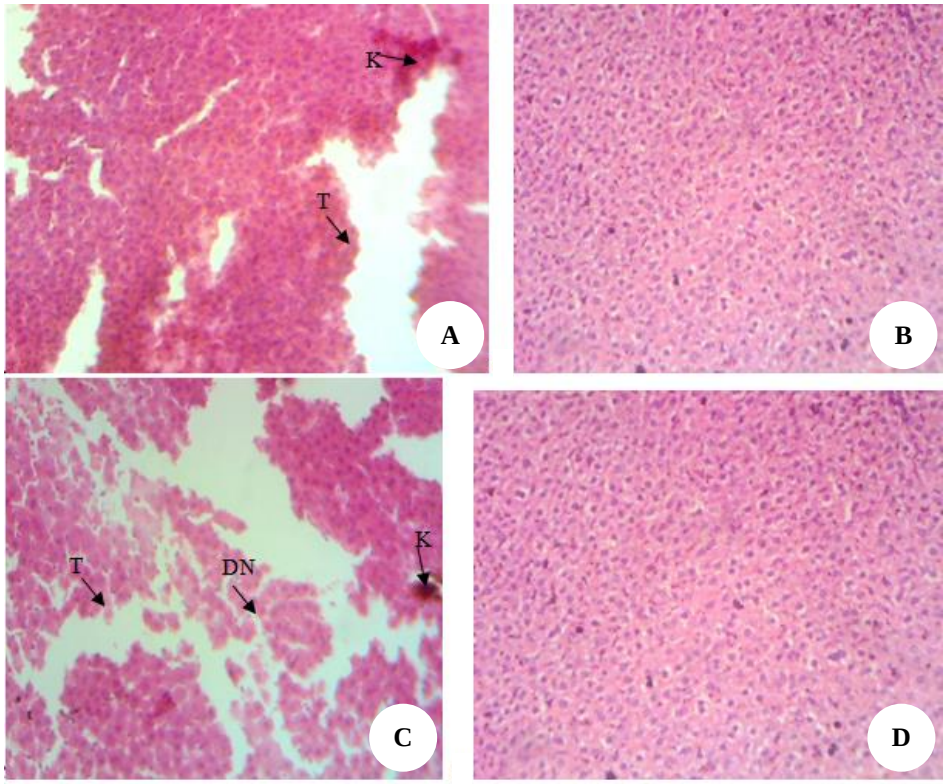

Figure 3. Photomicrograph of livers of rats fed unfermented and fermented castor seeds. A: fed raw castor seeds (BD+UCS), B: fed commercial fermented castor seeds (BD+CSF), C: fed castor seeds fermented with *Pleurotus ostreatus* (BD+CPF), D: fed basal diet (BD). Dilation of the sinusoid cells (T), degenerated hepatocytes (DN), and vascular congestion (K)

The increase in protein can be attributed to the presence of extracellular enzymes produced by *Pleurotus ostreatus*, which are proteins in nature (Alemawor et al., 2009; Sherief et al., 2010). These enzymes break down organic complexes to enhance the release and availability of proteins, which could contribute to the high protein content of the *Pleurotus ostreatus*-fermented castor seeds. The lowest protein content of CSF may be due to leaching during boiling. The lowest protein content may also be caused by the metabolic activities of the proteolytic microorganisms; they used the nitrogen content that contributed to the amount of protein content in the seed, thus, leading to the reduction in the protein contents of the locally fermented castor seeds (Ishiwu et al. 2015).

The fermented castor seeds' fiber contents were lower than the unfermented castor seeds. The lower fiber content could be of medicinal importance. The fiber reduction may be attributed to the ability of the fermenting microfloral to hydrolyze and metabolize castor seed (substrate) as a carbon source to synthesize cell biomass (Madigan et al. 2002). The fat contents in the fermented castor seeds were very low compared to the unfermented castor seeds, which could be attributed to the possible degradation of fat by lipase produced by microorganisms during the fermentation (Kpanja et al. 2016). The fat content in castor seeds deviated from what was reported by Aisha et al. (2013) and Mosquera-Artamonov et al. (2018). This could result from drying castor seed before fermentation and the difference in methods adopted.

The ash content of castor seeds fermented with *Pleurotus ostreatus* was the lowest compared to UCS and CSF. The reduction of ash content in castor seeds fermented with *Pleurotus ostreatus* may be due to leaching due to microbial activities that some of the minerals were lost or used *(*Kpanja et al. 2016). The high ash content of UCS may be due to the high content of minerals present in the seeds, which had been reported by Aisha et al. (2013) when they examined wild castor seeds from the Northern region of Nigeria.

The mineral composition: lead, nickel, chromium, and calcium were reduced in the fermented castor seeds (CSF and CPF) compared to unfermented (UCS). The finding of Ikanone and Oyekan (2014) revealed that boiling might cause the leaching of minerals into water, which may reduce the amount of these minerals in fermented samples. Although, the concentration of zinc, iron, magnesium, manganese, and copper was higher in the fermented castor seeds than in the unfermented castor seeds. This result may be due to releasing these elements from their complex states, thereby making them available in the fermented form (Omodara and Olowomofe 2013). Magnesium had the highest concentration in all the samples (UCS, CSF, and CPF); hence fermented castor seeds are a source of magnesium. Magnesium is a highly essential mineral for human growth; it plays an important role in forming teeth

and bones. In thermoregulation, it regulates calcium balance and acts as an activator of numerous enzyme systems (Dinu et al., 2014).

The phytochemical study of the unfermented and fermented castor seeds revealed the presence of phenol, saponins, alkaloids, flavonoids, and tannins. These phytochemicals in foods of plant origin indicate that they can be used as functional foods and for medicinal benefits (Settharaksa et al., 2012). This study reduced the flavonoid concentration in the fermented castor seeds, with CPF having the lowest concentration. Microbial enzymes, such as glucosidase, amylase, cellulase, tannase, esterase, invertase, or lipase produced during fermentation, are able to hydrolyze glucosides and break down plant cell walls matrix or starch and consequently facilitating the flavonoids extraction (Hur et al. 2014). During fermentation, the β-glucosidases of microbial origin could also be used to hydrolyze the phenolics and flavonoids (Nazarni et al. 2015), thereby reducing flavonoids.

The tannin concentration was reduced in the fermented castor seeds, with CPF having the lowest concentration (0.05 mg/L). Ejinkeonye et al. (2018) reported a similar trend when raw watermelon seeds were fermented to a local condiment called 'Ogiri.' Rodríguez et al. (2009) revealed that during fermentation, tannic acid degrades into smaller components such as glucose and gallic acid. The microorganisms involved during fermentation may also cause a reduction in tannin. According to Fan et al. (2000) and da Luz et al. (2013), *Pleurotus ostreatus* can eliminate antinutrients, mainly tannin, by the action of a tannase present in the *Pleurotus ostreatus*, which ultimately destroys the tannins.

Saponin was reduced in 'Ogiri,' a locally made condiment from castor seeds (CSF), while CPF has the highest concentration. Research carried out by Okwulehie et al. (2018) revealed that *Pleurotus ostreatus* has a higher content of saponin, which could have led to the high saponin content of CPF. The reduction observed in CSF may result from a long boiling time and fermentation. Heat processing and fermentation reduce phytochemicals/anti-nutritional concentration in raw food to an acceptable nutritional level (Oranusi et al., 2014).

There was no significant difference in the phenolic content of the UCS, CSF, and CPF. This result indicates that castor seeds and their fermented products are good candidates and sources of phenolic compounds. Phenols are commonly present in foods originating from the plant. Phenols protect plants from oxidative damage (Okwu 2005). It has been highly medicinal for different purposes, specifically blocking enzymes that cause inflammations and modifying the prostaglandin pathways, thereby protecting platelets from clumping (Okwu and Omodamiro 2005). Fermented castor seeds (CPF and CSF) had higher alkaloid content than raw castor seeds. Research carried out by Okwulehie et al. (2018) revealed that *Pleurotus ostreatus* has high alkaloid content, which may probably be responsible for the increased alkaloid concentration of CPF.

Glutamic acid was the most abundant amino acid, followed by aspartic acid, while glutamine and asparagine were absent in the samples (both raw and fermented samples). This trend corresponds with the observations of Khattab et al. (2009), who observed that glutamic and aspartic acid are the most abundant amino acids in some oilseeds and legumes. During the acid hydrolysis, glutamine and asparagine were converted to glutamic and aspartic acids with the liberation of ammonium (NH_4^+) ions, resulting in the high concentration of glutamic and aspartic acids and also the absence of glutamine and asparagine (Onwuliri and Anekwe 2001). The raw (unfermented) and fermented castor seeds may serve as a good source of monosodium glutamate due to the high concentration of glutamic acid since it is being used as a substrate for a condiment. Monosodium glutamate serves as a major component of food seasonings (Ishiwu et al., 2015). The reduction in protein content may result from boiling time before fermentation, which leads to the leaching of the amino acid content. Heat may lead to Maillard reactions, and subsequent Amadori rearrangements make the protein and its amino acids significantly unavailable (Igwe et al., 2012). It was observed that the total content of tryptophan, methionine, and cysteine as essential amino acids was enhanced after fermentation. The increase may result from the action of the microflora and enzymes, which break down chemical constituents and, thus, enhance the available amino acids (Igwe et al. 2012). Alemawor et al. (2009) reported that *Pleurotus ostreatus* are rich in essential amino acids, thereby increasing amino acids in CPF. This result agrees with Bao et al. (2013), who reported the nutritional properties of rice with *Pleurotus eryngii* and found the total content of essential amino acids enhanced after fermentation.

The rats fed UCS and CPF have lower PCV than those fed the basal diet. The decrease in PCV may result from the decreased RBC, which had been earlier reported by Etim et al. (2014). Rats fed CSF had similar PCV to rats fed the basal diet alone. This result suggests that CSF had a positive immunomodulatory effect. The rats fed UCS had the lowest hemoglobin, while the rats fed the basal diet had the highest hemoglobin. The reduced amount of hemoglobin in the groups fed UCS and fermented castor seeds (CPF) may indicate the varying degree of unstable aliment. This result agrees with Ekeh et al. (2010), which observed a reduction in the hemoglobin level of rats drunk with water contaminated with engine oil and suggested that the reduction is due to blood cell deficiency caused by the presence of the toxic substance. The rats fed raw and fermented castor seeds had lower RBCs than those fed the basal diet. The low RBC values may be due to their low hemoglobin concentration. RBCs are mainly made up of hemoglobin, which gives color to RBCs and helps transport oxygen throughout the body (Preet and Prakash 2011). The observed reduction in RBC may be attributed to the cytotoxic effects of compounds present in the castor seeds (Eyong et al., 2004). The increase in the lymphocyte count in rat-fed castor seeds could have been associated with the body's defense mechanism when exposed to a foreign substance, perhaps the residual compound in castor seeds. Lymphocytes help humoral antibody formation and cellular

immunity (Aboderin and Oyetayo 2006). Although, there was a relatively increased White Blood Cells (WBC) and lymphocyte count in rats fed fermented castor seeds, which could also suggest that the fermented castor seeds had an immunostimulatory effect because the rats were stable without any impediments observed. Oluwafemi et al. (2016) reported that *Pleurotus ostreatus* has high nutritional contents, which boosts the immune system of rats since it has been incorporated into the fermented product. The increase in the WBC counts of rat-fed CPF suggests that this group would have greater immunity to infections, thereby having an immunostimulatory effect. This result agrees with Nfambi et al. (2015), who observed an increase in the WBC count of immunosuppressed rats treated with methanolic leaf extracts of *Moringa oleifera*. There was a decrease in the neutrophil counts of the rats fed UCS, CSF, and CPF compared to the control. It may result from the amount of neutrophils used during sensitization to an antigen (Weber et al., 2015). The significant reduction in the hematological parameters of the rats fed UCS shows the hematotoxicity; a similar effect was reported by Eyong et al. (2004), who observed a significant reduction in the RBC values of rats and rabbits after ingestion of crude oil, an organic molecule containing a toxic compound (aliphatic and aromatic hydrocarbons), which result in the generation of free radical species in various tissues. In red blood cells, free radicals are known to alter the erythrocyte membrane as well as other cell membranes as a consequence of oxidative stress (Ita and Udofia 2011). The results of the liver histopathology revealed that the control group and CSF had the normal liver structure of healthy rats, which contains several hepatic lobules. Rats fed UCS and CPF had dilated sinusoid cells and degenerated hepatocytes. The kidney of the rats fed basal diet and CSF showed normal histological structures of the glomeruli and renal tubules. The kidneys of rats fed the basal diet, and CSF had normal kidney structure. In contrast, rats fed UCS and CPF showed various defects, such as shrunken glomerulus, hyalinization, enlarged renal tubules, and hemorrhagic lesions. It may be due to the effect of ricin, the toxic compounds present in the seeds. Ricin is quite stable and extremely toxic to the cells of different organs such as the liver, kidney, lung, pancreas, intestine, and thyroid (DaSilva 2003). Ingestion of ricin results in gastrointestinal hemorrhage, necrosis of the liver, spleen, and kidneys; severe localized muscle pain; regional lymph node necrosis, and moderate involvement of visceral organs (Olayioye et al. 2014). This result agreed with Hassan et al. (2015). They reported the effect of the acute phase of toxicity (in different doses) of aqueous extracts of castor seeds on the organs of albino rats. They observed that the internal organs showed degenerative changes and proteinaceous material in the spleen and kidney after 24 hours.

In conclusion, this study revealed that *Pleurotus ostreatus* was able to degrade the toxic compounds present in castor seeds to a greater extent. The results also revealed that *Pleurotus ostreatus* helped to enhance the nutritional properties of the fermented castor seeds. *Pleurotus ostreatus* could be used to detoxify castor seeds on a larger scale and reduce the boiling time of castor seeds before natural fermentation. *Pleurotus ostreatus* might be used in the detoxification of other poisonous plants.

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Antioxidant and alpha-glucosidase inhibitory activities of *Euchresta horsfieldii*

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Abstract. *Prihantini AI, Krisnawati, Setyayudi A. 2019. Antioxidant and alpha-glucosidase inhibitory activities of* Euchresta horsfieldii. *Biofarmasi J Nat Prod Biochem 17: 65-68. Euchresta horsfieldii*, known as *pranajiwa*, is a medicinal plant widely grown in Bali and West Nusa Tenggara, Indonesia. Its seeds or fruits are commonly used for body freshness and stamina. The present study aimed to investigate the biological activities of leaves, roots, stems, fruits, and seeds of *E. horsfieldii*. Antioxidants, alpha-glucosidase inhibitory activities, and total phenolic compounds were evaluated from methanolic extracts of all parts of *E. horsfieldii*. The result showed that leaf extract of *E. horsfieldii* exhibited the highest antioxidant activity with IC_{50} 215.11±08.06 µg/mL. Meanwhile, the root extract had the highest alpha-glucosidase inhibitory activity and total phenolic compound with IC_{50} 29.76±13.17 µg/mL and 763±0.01 mg GAE/100mg dry extract, respectively. In conclusion, the study suggested that *E. horsfieldii* is the potential as a natural source of antioxidant and alpha-glucosidase inhibitor agents.

Keywords: Alpha-glucosidase inhibitor, antioxidant, *Euchresta horsfieldii*, total phenolic content

INTRODUCTION

Free radicals, chemical compounds having one or more unpaired electrons, play a critical role in our health. They are highly unstable and can damage other molecules by extracting electrons in order to attain stability. Reactive oxygen species (ROS), the free radical generated from oxygen, initiate bimolecular oxidation and affect resultant DNA damage, protein carbonylation, and lipid peroxidation, which leads to cell death and creates oxidative stress such as cancer, cardiovascular disease, neurological disorder, arthritis, atherosclerosis as well as aging process (Khalaf et al. 2007; Pham-Huy et al. 2008; Chanda and Dave 2009; Piao et al. 2009). Antioxidants can help our body deal with oxidative stress caused by free radical-induced damage.

The high accumulation of ROS can also enhance the risk of diabetes (Ha et al. 2008; Noh and Ha 2011). Diabetes mellitus is a condition defined by the level of hyperglycemia. Some reports suggest that decreasing the level of postprandial hyperglycemia is one of the most effective therapeutic approaches in the early period of diabetes mellitus (Lee et al. 2009; Ghadyale et al. 2011; Van de Laar et al. 2012). Other studies demonstrated that alpha-glucosidase inhibitors helped control postprandial blood glucose levels in diabetic patients (Sancheti et al. 2011; Gurudeeban et al. 2012).

Our nature is rich in potential compounds for drugs or medicines. In Indonesia, a medicinal plant is known as *pranajiwa* (*Euchresta horsfieldii)* grows in forests and is categorized as a threatened plant (Mogea et al. 2001). Its seeds and fruits have been traditionally used for stimulants, aphrodisiacs, refreshments, asthma, tuberculosis, cough, hyperlipidemia, etc. (Heyne 1987; Kloppenburgh 2006; Tirta et al. 2010; Kim et al. 2011). The seeds and fruits are produced as a traditional herbal drink called jamu and other products with high economic value. The leaf of *E. horsfieldii* has been reported to have antioxidant activity and increase enzyme activity of superoxide dismutase and glutathione peroxidase in Wistar rats (Tirta et al. 2010; Gunawan et al. 2017). However, fewer reports studied other parts of the plant and alpha-glucosidase inhibition activity as an approach for diabetes management. Therefore, the aim of the study was to evaluate the bioactive compounds, particularly antioxidants and alpha-glucosidase inhibitory activities, from roots, stems, leaves, fruits, and seeds of *E. horsfieldii*. Furthermore, the total phenolic content of the extracts was also evaluated since they are considered to contribute as antioxidants or alpha-glucosidase inhibitors.

MATERIALS AND METHODS

Chemicals

Quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), *p*-nitrophenyl-α-D-glucopyranoside, and alpha-glucosidase were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan. Folin-Ciocalteu reagent was purchased from Sigma Aldrich, Japan. All the solvents used were at the highest purity available.

Extract preparation

Approximately 10 g dried samples of roots, stems, leaves, fruits, and seeds of pranajiwa were extracted with distilled methanol. The extracts were filtered and concentrated under a rotary evaporator and then vacuumed and dried. The extraction was conducted twice to obtain approximately 1 g crude extract.

DPPH radical scavenging activity assay

The DPPH radical scavenging activity was conducted with minor modifications based on a previous method (Jayaprakasha et al. 2001). Various concentrations at 20, 50, 100, and 200 µg/mL of samples were mixed with 0.5 mL of methanol 1 mM DPPH radical solution. A similar solution without a sample was used as a control. Absorbance was measured using a UV-vis spectrophotometer at 517 nm after incubation at room temperature under dark conditions for 30 min. Quercetin was used as a positive standard. The following formula determined the percentage of scavenging activity:

Scavenging activity (%) = [($A_{control}$ - A_{sample})/$A_{control}$] x 100%

Alpha-glucosidase inhibition assay

The alpha-glucosidase inhibitory activity of the crude extracts was evaluated using a method described by Dewi et al. (2014). The reaction mixture containing 5 µL of the sample was dissolved in DMSO at various concentrations, and 495 µL of 100 mM phosphate buffer (pH 7.0) was added to 250 µL of 3 mM substrate solution *p*-nitrophenyl-α-D-glucopyranoside (PNPG). The mixtures were pre-incubated for 5 min at 37ºC, and 250 µL of alpha-glucosidase (0.065 unit/mL) was added to start the reaction. The incubation was continued for 15 min, and the reaction was terminated by adding 1 mL of 0.1 M sodium carbonate. Alpha-glucosidase inhibitory activity was quantified by measuring the released product of *p*-nitrophenyl at 400 nm. Quercetin was used as a positive standard.

Total phenolic content

The extracts' total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent (Singleton et al. 1999). Approximately 500 µL of the extracts (1.0 mg/mL) was added with distilled water, made up to 8 mL, and then mixed with 500 µL of 2 N Folin-Ciocalteu reagents. The mixture was allowed to stand for 8 min, and 1.5 mL of 20% sodium carbonate was then added. The reaction mixture was incubated at room temperature for 2 h. Absorbance was measured at 765 nm, and the phenolic content was determined using a calibration curve obtained from gallic acid.

Statistical analysis

All assays were performed in triplicates, and the data were expressed as the mean ± standard deviation. Statistical analysis was carried out using SPSS version 16.0 for Windows, followed by Tukey`s post hoc test. Differences at $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH is a stable radical widely used to evaluate the activity of scavenging free radicals. Free radicals were reduced by antioxidants that donate their hydrogen (El-Haci et al. 2013). Therefore, DPPH radical scavenging assay is a common method for antioxidant evaluation. The discoloration measured the antioxidant activity to yellow as a stable molecule 2,2-diphenyl-1-hydrazine formed from purple solution as DPPH radicals in methanol. Activity on scavenging DPPH radical of the extracts of pranajiwa is shown in Figure 1. The dose-dependent activity is shown as an IC_{50} value reflecting the required concentration for 50% inhibition. A lower IC_{50} value represented higher antioxidant activity.

Leaves extract showed the highest activity, followed by roots and stem extracts with IC_{50} 215.11±8.06, 266.18±13.17, and 302.67±26.90 µg/mL, respectively. Meanwhile, fruits and seeds extracts had a lower activity with IC_{50} 462.7±38.32 and 468.79±42.27 µg/mL, respectively. Interestingly, besides fruits and seeds, which Indonesian people usually consume, the leaves revealed potency as a medicinal source. These results were supported by Sari et al. (2015), who reported that *n*-hexane leaves extract of pranajiwa had high antioxidant activity. Therefore, the antioxidant potency of its leaves can be considered for further utilization of herbal medicine.

The strength of antioxidant activity depends on the existence of various secondary metabolites (Emami et al. 2007). Some phenolic compounds, tannins, phenyl isopropanoids, lignans catechol, and many others are good antioxidants (Rice-Evans et al. 1996). Several essential oils have also been studied for their antioxidant activities (36). Variation in the amounts of various non-volatile and volatile compounds can be one of the reasons causing differences in the antioxidant activity of the extracts from different parts of the plant (Emami et al. 2007).

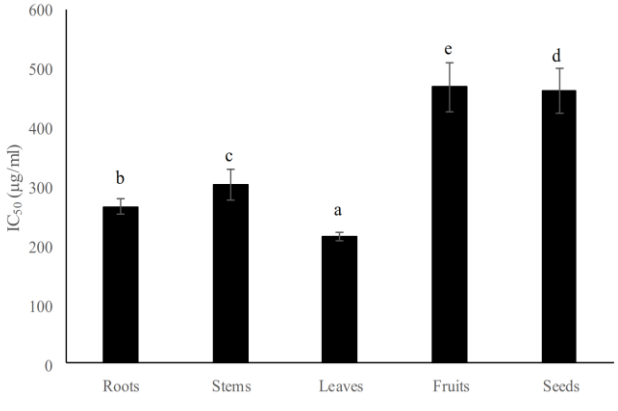

Figure 1. Antioxidant activity of pranajiwa (*Euchresta horsfieldii*) methanolic extracts

Alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibition assay can be related to the effort to search for alternative medicines, particularly for type 2 diabetes mellitus (DM). An approach to treat type 2 DM is by reducing blood glucose levels in the body. Alpha-glucosidase is a catalyzing enzyme in the final digestion process of carbohydrates. The enzyme breaks down oligosaccharides and disaccharides into monosaccharides. Delaying absorption of carbohydrates is necessary for glycemic control (Cheng and Josse 2004). Inhibiting alpha-glucosidase activity retards the absorption of glucose (Ghadyale et al. 2011; Lee et al. 2009; Van de Laar et al. 2005). Therefore, the inhibition of alpha-glucosidase activity can be used in diabetic management.

Alpha-glucosidase inhibitory activity of the extracts of pranajiwa is shown in Table 1. Roots extract had the highest activity against alpha-glucosidase, followed by stems extract with IC_{50} 29.76±3.76 and 107.95±4.68µg/mL, respectively. Meanwhile, leaves, fruits, and seeds extract of pranajiwa did not show alpha-glucosidase inhibition. Roots extract, which showed high antioxidant activity and highest alpha-glucosidase inhibitory activity, revealed strong potency as a natural source of the anti-diabetes agent. It is due to that an ideal anti-diabetes treatment should possess both hypoglycemic and antioxidant properties (Shibano et al. 2008). It is also indicated that an antioxidant assay combined with an alpha-glucosidase inhibition assay is necessary to consider the potencies of the plant in diabetic treatment.

Total phenolic content

Phenolic compounds are commonly considered to play an important role in antioxidant and alpha-glucosidase inhibitory activities. Therefore, it is necessary to evaluate total phenolic content to support antioxidant and alpha-glucosidase inhibition assays. The phenolics content was expressed in the percentage of the gallic acid equivalent (GAE). The evaluation of total phenolic content from various extracts of pranajiwa revealed that roots extract had the highest phenolic contents (7.63±0.01 %GAE/dry extract). On the other hand, leaves, stems, and seeds are slightly lower than roots extract (7.63±0.01; 6.03±0.09; 5.89±0.01; and 4.09±0.11 %GAE/dry extract), as shown in Table 2.

Phenolic compounds can donate their electron without becoming reactive radicals; therefore, they are considered good antioxidants. As the previous result showed that the leaves and stems had higher antioxidant activity, it implied that their phenolic contents contributed to the antioxidant activity. Due to the chemical structures of phenolics, they can stabilize unpaired electrons (Chanda and Dave 2009).

Roots extract showed the highest alpha-glucosidase inhibitory activity and higher antioxidant activity, indicating its high contribution of phenolic content. Meanwhile, an ideal anti-diabetes treatment should possess both antioxidant and hypoglycemic properties (Shibano et al. 2008). Therefore, the evaluation of antioxidants combined with alpha-glucosidase inhibition assays is an approach that can be considered a strategy for the management of diabetes. The free radicals generated from oxygen, reactive oxygen species (ROS), can cause excessive oxidation and affect diabetes (Basma et al. 2011; Meziti et al. 2012). ROS production increases under diabetic conditions (Noh and Ha 2011; Ha et al. 2008). Meanwhile, alpha-glucosidase inhibitors helped control postprandial blood glucose levels in diabetic patients (Sancheti et al. 2011; Gurudeeban et al. 2012). It reduces digestion and delays carbohydrate absorption, relieving the postprandial increase in blood glucose levels (Cheng and Josse 2004). Therefore, a root extract of pranajiwa showed potency as a source of the antidiabetic agent. However, further study on the isolation of the active compounds is required.

On the other hand, leaf extract did not become active against the alpha-glucosidase enzyme. Still, it revealed that antioxidant activity and total phenolic content were slightly lower than root extract. The result implied that the phenolics might contribute to antioxidant activity; however, there is less support for alpha-glucosidase inhibitory activities. It is considered that other secondary metabolites, such as alkaloids and terpenes, might play a role in alpha-glucosidase inhibitory activities, as reported by a previous study (Bahmani et al. 2014). In addition, antagonistic or synergetic interactions between secondary metabolites may have contributed to the activity (Prihantini et al. 2014; Bahmani et al. 2014).

In conclusion, the leaves, roots, and stems of pranajiwa had potency for antioxidants. Furthermore, the roots had the highest activity against alpha-glucosidase enzyme and total phenolic content. The study suggested that the leaves, roots, and stems of pranajiwa had high potency as a medicinal source. Therefore, the overexploitation of seeds or fruits of pranajiwa can be reduced. Studies on the isolation of the compounds responsible for the antioxidant and or alpha-glucosidase inhibitory activities of pranajiwa are warranted.

Table 1. Alpha-glucosidase inhibitory activity of pranajiwa extracts

Extracts	IC_{50} (µg/mL)
Roots	29.76±3.76
Stems	107.95±4.69
Leaves	n.a
Fruits	n.a
Seeds	n.a

Note: n.a = not active

Table 2. Total phenolic content of pranajiwa (*Euchresta horsfieldii*) methanolic extracts

Extracts	Total phenolic content (%GAE/dry extract)
Roots	7.63±0.01[a]
Stems	5.89±0.01[c]
Leaves	6.03±0.09[b]
Fruits	1.67±0.05[e]
Seeds	4.09±0.11[d]

Note: Data were expressed as mean ± standard deviation. Different letters in the same column indicate significant differences ($p<0.05$). GAE = Gallic acid equivalent

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Antibacterial activity of ethanolic and n-hexane extracts of *Ruellia tuberosa* leaves against *Escherichia coli* and *Bacillus subtilis* bacteria

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Abstract. *Amijida H, Purwoko T, Susilowati A. 2019. Antibacterial activity of ethanolic and n-hexane* extracts of Ruellia tuberosa *leaves against* Escherichia coli *and* Bacillus subtilis bacteria. *Biofarmasi J Nat Prod Biochem 17: 69-80. Ruellia tuberosa* L. has pharmacological activity, one of which is antibacterial. This study aimed to determine the antibacterial activity and the minimum inhibitory concentration (MIC) of pletekan leaf extract (*R. tuberosa*) against *Escherichia coli* and *Bacillus subtilis* and the chemical compounds contained in the leaves of *R. tuberosa*. This study used a completely randomized design with five treatments and three replications. *Ruellia tuberosa* leaf extract was obtained by extraction by maceration and reflux using ethanol and n-hexane as solvents. The concentrations of the *R. tuberosa* leaf extract used were: P1 (125 mg/mL), P2 (250 mg/mL), P3 (500 mg/mL), P4 (1000 mg/mL) and P5 (2000 mg/mL) with dimethylsulfoxide (DMSO) solvent. The positive control was in a 30 g chloramphenicol antibiotic disc, while the negative control used was DMSO solvent. The agar diffusion method used a paper disc to conduct the antibacterial activity test. The minimum inhibitory concentration of *R. tuberosa* leaf extract was determined using the dilution method. Identification of secondary metabolites was carried out using phytochemical screening. The data obtained were analyzed using the One-Way ANOVA test and if there was a significant difference, then continued with the Tukey test. *Ruellia tuberosa* leaf extract with ethanol solvent has better antibacterial activity against *E. coli* and *B. subtilis* than n-hexane. The minimum inhibitory concentration of *R. tuberosa* leaf ethanol extract against *E. coli* was 500 mg/mL with a 99.1% inhibition percentage. The minimum inhibitory concentration of *R. tuberosa* leaf ethanol extract against *B. subtilis* is 1,000 mg/mL with a 99% inhibition percentage. The ethanolic extract of *R. tuberosa* leaves contains alkaloids, flavonoids, tannins, saponins, and glycosides, while the n-hexane extract of *R. tuberosa* leaves contains terpenoids.

Keywords: Antibacterial, *Bacillus subtilis, Escherichia coli,* phytochemical screening, *Ruellia tuberosa*

INTRODUCTION

Antibacterial is a substance that inhibits or kills the growth of microorganisms. Microorganism growth control aims to prevent disease and infection from spreading, eradicate microorganisms in infected hosts, and prevent spoilage and destruction of materials by microorganisms (Setyaningsih et al., 2012). Antibacterial power was measured in vitro to determine the ability of an antibacterial substance. The phenomenon of natural plant resistance to bacteria has led to the development of several compounds derived from plants with antibacterial and antifungal properties (Jawetz et al., 2008).

Chemical compounds resulting from secondary metabolites have been widely used as dyes, poisons, food aromas, and medicines. Various plants are used as traditional medicines, so research is needed on efficacious plants and the chemical compounds that function as drugs (Apristiani and Astuti 2005; Lenny 2006). A solvent can pull the compounds' content in plants during the extraction process. Selecting a suitable solvent is an essential factor in the extraction process. The solvent used in the extraction determines the quality of the simplicia, namely the composition of the compounds contained in the extract. Ethanol is often used as a solvent in the laboratory because it has relatively high solubility and is inert, so it does not react with other components. Ethanol is a polar solvent, so the active components contained in plants can be extracted more completely. Meanwhile, *n*-hexane is the lightest solvent in the extraction process and evaporates quickly, making it easier for reflux (Susanti et al., 2012).

Based on the cell wall composition, bacteria are divided into two, namely Gram-positive and Gram-negative bacteria. Gram-positive and Gram-negative bacteria cell walls are composed of a peptidoglycan polymer with elastic peptide-glycan cross-links. The resulting elastic tissue protects the cell from lysis. Gram-positive bacteria have a thick layer of peptidoglycan, while Gram-negative bacteria have a thin layer of peptidoglycan but have an additional membrane, namely the outer cytoplasmic membrane. This outer membrane layer functions as an additional permeability barrier and is used as a mechanical transport membrane (Pelczar and Chan 2008).

Ruellia tuberosa L. is traditionally used to treat diuresis, antidiabetic, antipyretic, antihypertensive, and antidote agents. In Taiwan, *R. tuberosa* is a simplicia added to health drinks (Lin et al. 2006). In Trinidad and Tobago, *R. tuberosa* is used as an antihypertensive and cooling agent (Lans 2006). This plant has not been widely used in Indonesia; even better known as a weed. Increasing its use requires scientific studies regarding this plant's biological or pharmacological activities (Ahmad 2012).

Several chemical constituents and antimicrobial activity have been reported for *R. tuberosa* from Taiwan and India. Chloroform, ethyl acetate, alcohol, and aquadest extracts from the whole plant *R. tuberosa* showed significant antibacterial properties. The distilled water extract showed less activity against fungal organisms (Arirudran et al., 2011).

The methanolic extract of *R. tuberosa* root showed significant antibacterial and antifungal properties with an inhibition zone of 9-23 mm for antibacterial screening and 8-15 mm for antifungal screening. Tests of insecticides using the surface film activity test showed strong insecticidal activity with a mortality rate of 80% of *Tribolium castaneum* (Herbst) at a 50 mg/mL dose in 48 hours (Kader et al. 2012).

The methanolic extract of the leaves of *R. tuberosa* showed significant antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Proteus mirabilis*, and antifungal activity against *Aspergillus* sp., *Mucor* sp., *Penicillium* sp. and *Fusarium* sp. The antibacterial potential of the methanolic extract of *R. tuberosa* was tested using the agar diffusion method. A total of 100 mg/mL of leaf extract showed the maximum zone of inhibition against *P. mirabilis* (7mm) and *Aspergillus* sp. (8 mm) (Vasantharaj et al., 2013). From the literature review, it is known that *R. tuberosa* has antibacterial activity. Therefore, this study was conducted to increase its utilization to determine the power of antibacterial leaf extract of *R. tuberosa* against the growth of *E. coli* and *B. subtilis.*

The aims of this study were: (i) To determine the antibacterial activity of pletekan leaf extract (*R. tuberosa*) with ethanol and n-hexane as solvents against *E. coli* and *B. subtilis.* (ii) Knowing the minimum inhibitory concentration (MIC) of *R. tuberosa* leaf extract with ethanol and n-hexane as solvents against *E. coli* and *B. subtilis.* (iii) Knowing the chemical compounds contained in the leaf extract of *R. tuberosa* with ethanol and *n*-hexane as solvents.

MATERIALS AND METHODS

Research time and place

The study held from December 2015 to March 2016 took place at the Laboratory of Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret (UNS), Surakarta, Sub-laboratory Chemistry UNS Surakarta, Central Java, Indonesia, and Laboratory of Food and Nutrition Culture Collection (FNCC) Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia.

Ingredient

The materials used in this study included: isolates of *E. coli* bacteria IFO 3301, *B. subtilis* bacterial isolate IFO 13719, and leaves of *R. tuberosa* obtained from Surakarta (Solo), Central Java, Indonesia.

Research design

This research uses a completely randomized design (CRD) with five treatments, namely variations in the concentration of *R. tuberosa* leaf extract with ethanol and n-hexane solvent, positive control in the form of chloramphenicol antibiotic disc 30 ug, and the negative control was DMSO solvent.

Procedures

Material preparation

The fresh leaves of the *R. tuberosa* plant were washed with running water until clean, drained, and air-dried so that they were free of the remaining water. *R. tuberosa* leaves were blended for ± 2 minutes so that the samples of *R. tuberosa* leaves were smooth.

Sterilization

The equipment to be used must be sterilized first to avoid contamination. The sterilized equipment includes Petri dishes, Erlenmeyer, test tubes, beakers, stirring rods, and ose needle. Materials that must be sterilized are Nutrient Agar media and Mueller Hinton media. Sterilization is carried out using an autoclave at a temperature of 121^{o}C, a pressure of 1 atm for 15 minutes.

Extraction

The leaves of the *R. tuberosa* plant were cleaned and then aerated to dry, with a moisture content of 5%. The dried leaves are cut into pieces and blended until a powder is formed. A total of 300 g of powder in a 2,000 mL Erlenmeyer was macerated in 96% ethanol until all the powder was submerged (1,500 mL). Maceration was carried out for 6 hours while shaking using a shaker at 40 rpm. The soaked leaf powder of *R. tuberosa* was refluxed for 3 hours and filtered using Whatman No. filter paper. 42. The filtering dregs were refluxed with 96% ethanol and repeated two times. The ethanol contained in the filtrate was removed by evaporation using a vacuum evaporator at 40^{o}C so that a thick extract of 96% ethanol was obtained. The ethanol dregs were extracted again using n-hexane by refluxing two times and filtered using Whatman filter paper No.42. The resulting filtrate was removed by evaporation using a vacuum evaporator at 40^{o}C to obtain a thick extract of n-hexane. The yields obtained were weighed and recorded (Age et al. 2002; Harborne 2006).

$$\% \text{ extract content} = \frac{extract\ weight\ obtained\ (g)}{extracted\ sample\ weight\ (g)}\ x\ 100\%$$

Extract moisture test

The water content test was carried out to determine the remaining water in the extract. The procedure for testing the moisture content of the extract was as follows: 1 g of ethanolic extract and n-hexane were put into a porcelain dish that had been tared separately, then dried in an oven at 105^{o}C for 5 hours, followed by drying at a distance of 1 hour until the difference between 2 weights in a row, not more than 0.25% (Indonesian Ministry of Health 2000).

$$\% \text{ drying shrink} = \frac{b-c}{b-a}\ x\ 100\%$$

Where:
a = weight of the empty cup
b = weight of sample and cup before drying in the oven
c = weight of sample and cup after drying in the oven

Media preparation

Nutrient agar powder was weighed as much as 2.3 g and mixed with 100 mL of distilled water in an Erlenmeyer, then heated and stirred using a stirrer on a hotplate until dissolved. With the same treatment, 0.8 g of Nutrient Broth (NB) was mixed with 100 mL of distilled water in an Erlenmeyer, then heated and stirred using a stirrer on a hotplate until dissolved.

Preparation of test extract concentration

In this study, the concentration series of the test extracts (ethanol and n-hexane) used were 2000, 1000, 500, 250, and 125 mg/mL with dimethylsulfoxide (DMSO) solvent. The 2000 mg/mL test extract was prepared by weighing 400 mg of the extract and dissolved in 0.2 mL of DMSO. Concentrations of 1000, 500, 250, and 125 mg/mL were made by serial diluting the extract with DMSO. Sterile disc paper was saturated with 10 L of the test extract solution and dried in a sterile petri dish at room temperature.

Positive control and negative control

The positive control used was chloramphenicol antibiotic disc 30 g, while the negative control used was DMSO solvent (Natheer et al. 2012).

Bacteria suspension manufacturing

One ose of bacterial colonies was inoculated in 10 mL of NB and then incubated at 37ºC for 24 hours. The optical density of the culture was measured using a spectrophotometer at a wavelength of 625 nm (OD_{625}). The resulting OD_{625} is then converted to 0.1. OD_{625} 0.1 is equivalent to 0.5 McFarland standard (bacterial cell density $1x10^8$ cells/mL). The bacterial suspension was then diluted into a bacterial suspension with a cell density of 10^6 cells/mL by putting 1 mL of a 108 cell/mL bacterial suspension into a test tube containing 9 mL. NB was then vortexed, and a bacterial suspension was produced with a cell density of 10^7 cells/mL. Then 1 mL of bacterial suspension of 10^7 cells/mL was put into a test tube containing 9 mL of NB, vortexed, and a bacterial suspension with a cell density of 106 cells/mL was produced (Lopez et al. 2003; Widiastomo and Wahyu 2013).

Antibacterial test procedure

A total of 1 mL of bacterial suspension with a cell density of 106 cells /mL was poured into 15 ml of Nutrient agar that had solidified, then leveled using a glass rod. Preparation of media and pouring of bacterial suspension was carried out near the bunsen burner in Laminar Air Flow. After the agar solidified, each petri dish was made a 7-part diagram. Positive control paper discs, negative control discs, and discs saturated with extract solution were placed in each section and then incubated at 37ºC for 24 hours (Nufailah et al. 2008). This antibacterial test was repeated three times. The clear area around the disc indicated the inhibition of bacterial growth, which was then measured using a caliper. The diameter and area of the inhibition zone were calculated.

The formula calculates the diameter of the inhibition zone (D):

D = Inhibitory zone diameter – paper disc diameter

$$\mathrm{r} = \frac{D}{2}$$

Furthermore, the area of the inhibition zone (L) was calculated using the formula:

$L = \pi\ r^2$
π = constant
r = radius of inhibition zone (mm)

The results of the inhibition zone measurements were classified according to Table 1.

Minimum Inhibitory Concentration (MIC) of *R. tuberosa* leaf extract was determined by tube dilution test by decreasing the extract concentration (to close the dose) based on the diameter of the inhibition zone formed in the diffusion test. A total of 4 mL of sterile NB media was put into each test tube, added by extract according to the concentration of 0.5 mL. Subsequently, 0.5 mL of bacterial suspension was added to this medium at 106 cells/mL, adjusted to the 0.5 Mc standard Farland (repeated three times). Then the tubes were incubated for 18-24 hours at 37°C in an incubator (Fatisa 2013).

All variations in the concentration of the extract were compared with bacterial control and media control by looking at the turbidity of the test sample. The concentration of *R. tuberosa* leaf extract against bacteria showing clear color was then replanted on NB media to determine if the extract was bacteriostatic or bactericidal.

Data analysis of the liquid dilution method results was carried out by calculating the minimum inhibitory concentration value. The formula used to calculate the inhibition of bacterial growth is as follows (Quave et al. 2008):

$$\% \text{ inhibition} = \left[\frac{sample\ OD - blank\ sample\ OD}{DMSO\ OD - blank\ DMSO\ OD}\right] x\ 100\%$$

Where:
Sample OD: Optical density extract with suspension
Blank sample OD: Optical density extract with saline
DMSO OD: Optical density DMSO with microbial suspension
Blank DMSO OD: Optical density DMSO with saline

Phytochemical screening

Phytochemical screening was carried out on ethanol and *n*-hexane extracts. This test was conducted to determine the secondary metabolites of alkaloids, flavonoids, tannins, terpenoids, saponins, and glycosides. The test procedure is as indicate in Table 2.

The expansion vessel is saturated with the appropriate mobile phase for each class of active compound. The thick extract of the leaves of *R. tuberosa* was spotted on the layer chromatography (TLC) plate, and then the TLC plate was immersed in the developer's vessel. After that, the TLC plate was eluted, dried, and then detected with the appropriate spectrophotometer for each group of compounds present in the thick extract of *R. tuberosa* leaves. It was then observed in visible light and calculated its Rf (Wagner et al. 1984).

Data analysis

The diameter of the clear zone around the paper disc in each extract was analyzed descriptively with the help of tables and figures. The analysis used is a one-way ANOVA statistical test. One way ANOVA test was used to determine the effect of giving various concentrations of *R. tuberosa* leaf extract on the growth of *E. coli* bacteria and *B. subtilis.* If there is a difference, it is continued using the Tukey test.

RESULTS AND DISCUSSIONS

***Ruellia tuberosa* leaf extraction with ethanol and n-hexane solvents**

The technique of isolating secondary metabolites from natural materials is known as extraction. *R. tuberosa* leaves were extracted by maceration and reflux to extract the chemical components contained in Simplicia using ethanol and n-hexane as solvents. Each character, weight, and water content of the extract is presented in Table 3.

Based on the general standard parameters of plant extracts from the Ministry of Health of the Republic of Indonesia (2000), the water content limit of the extract is <10%. It indicates that the water content of the ethanol extract and n-hexane corresponds to the standard parameters of the extract water content. The quality of extracts produced from plant materials largely depends on the solvent used in the extraction procedure. Good solvent properties in plant extraction include low toxicity, volatility at low temperatures, and the ability to dissolve bioactive compounds contained in natural ingredients (Anand et al., 2015).

Table 1. Classification of antibacterial activity (Grenwood 1995)

Inhibition zone diameter (mm	Antibacterial activity
10	Not active
11-15	Weak
16-20	Currently
20	Strong

Table 3. Character *R. tuberosa* leaf extract with ethanol and *n*-hexane as solvent.

Extract	Ethanol	n -Hexane
Character		
Form	Thick extract	Thick extract
Color	Dark chocolate	blackish green
Smell	Typical	Typical
Extract weight		
Weight (grams)	30.35	4.89
Yield (%)	10,12	1.63
Water content		
Initial weight (g)	20, 3333	20.9407
Final weight (g)	20.2742	20.8719
Water content (%)	5.74	7.16

Note: Initial weight: The weight of the sample and crucible before drying in the oven; Final weight: Weight of sample and cup after drying in the oven

Table 2. Chromatographic characteristics

	Alkaloids	Flavonoids	Tannins	Terpenoids	Saponins	Glycoside
Silent phase	Silica Gel 60 F254	Silica Gel 60 F254	Silica Gel 60 F254	Silica Gel 60 F254	Silica Gel 60 F254	Silica Gel 60 F254
Mobile phase	Toluene: ethylacetate: diethylamine (7:2:1)	Ethylacetic: formic acid: acetic acid glassial: water(100:11:11:27)	chloroform: ethylacetate: formic acid (0.5:9:0.5)	Hexane: ethylacetate (93:7)	Chloroform:methanol:water (64:50:1)	Ethylacetic: formic acid: toluene: water (6:1.5:3:0.5)
Comparison	Quinine 10 mg/ 1 mL ethanol	Routine 10 mg/1 mL ethanol	Tannins 10 mg/ 1 mL ethanol	Tymol 10 mg/1 mL ethanol	Saponins 10 mg/1 mL ethanol	Gallic acid 10 mg/1 mL ethanol
Detection	Dragendor (viewed after sprayed on visible light orange)	cytroborate (viewed after sprayed on visible light-colored yellow)	$FeCl_3$ (viewed after sprayed on the light looks colorful dark green-black)	Anisaldehyde sulfuric acid (viewed after being sprayed on the light looks colored Red purplish)	Lieberman Bourchat (viewed after sprayed on visible light (blackish brown)	$FeCl_3$ (seen after being sprayed on visible light is dark green-black)

Antibacterial activity of *R. tuberosa* leaf extract with ethanol and n-hexane

Antibacterial is a typical chemical compound produced by living organisms in low concentrations and can inhibit essential processes in a microorganism. Disc diffusion test is carried out by measuring the diameter of the clear zone, which indicates a response to inhibition of bacterial growth by an antibacterial compound in the extract (Hermawan et al. 2007). The antibacterial activity of *R. tuberosa* leaf extract with ethanol and n-hexane as solvents against *E. coli* and *B. subtilis* bacteria can be seen in Figure 3.

Table 4 shows that the ethanolic extract of the leaves of *R. tuberosa* against *E. coli* had a larger diameter of inhibition zone than *B. subtilis.* Based on the classification of antibacterial activity by Grenwood (1995), a concentration of 2000 mg/mL of ethanolic extract of *R. tuberosa* leaves against *E. coli* had weak antibacterial activity. On the other hand, it had no antibacterial activity or was inactive for other concentrations because the diameter of the inhibition zone was 10 mm. The inhibition zone diameter formed in the ethanol extract of the leaves of *R. tuberosa* against *B. subtilis* did not show any antibacterial activity because the inhibition zone diameter was 10 mm.

Table 5 shows only the concentration of 2,000 mg/mL of n-hexane leaf extract against *E. coli* which resulted in the presence of an inhibition zone, while for *B. subtilis* at all variations of extract concentration did not produce an inhibition zone. Based on the classification of antibacterial activity by Grenwood (1995), n-hexane extract against *E. coli* and *B. subtilis* did not have an antibacterial activity or was inactive because the diameter of the inhibition zone was 10 mm. Based on Table 4 and Table 5, the comparison of the diameter of the inhibitory zone of *R. tuberosa* leaf extract with ethanol and n-hexane solvents against *E. coli* bacteria and *B. subtilis* can be seen on the next curve (Figure 4A-D).

Figure 4 shows the diameter of the inhibition zone of the ethanolic extract of the leaves of *R. tuberosa* against *E. coli* was greater than that of *B. subtilis*, which means that the ethanol extract had more significant antibacterial activity against *E. coli* than *B. subtilis.* Meanwhile, the n-hexane extract of *B. subtilis* did not show any inhibition zone, in contrast to *E. coli* which only shows the diameter of the inhibition zone at a concentration of 2000 mg/mL (Figure 4B). Figure 4C and 4D show that the diameter of the inhibition zone of the ethanolic extract of *R. tuberosa leaves* against *E. coli* and *B. subtilis* was greater than that of the *n*-hexane extract. It means that the ethanol extract had more significant antibacterial activity than *n*-hexane extract in inhibiting the growth of *E. coli.* and *B. subtilis.*

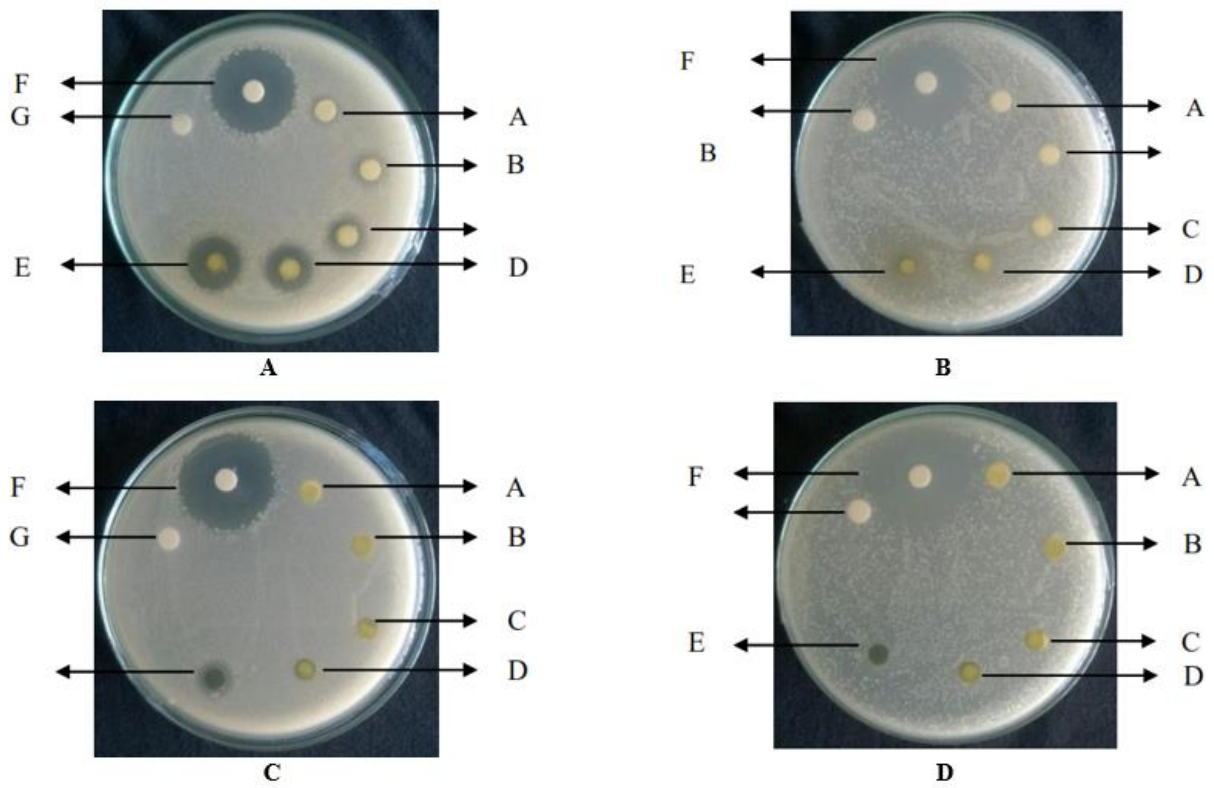

Figure 3. A. Diameter resistor extracts ethanol leaf *R. tuberosa* to bacteria *E. coli* (A) and *B. subtilis* (B). Inhibitory diameter of *n-hexane* extract of leaves of *R. tuberosa* to bacteria *E. coli* (C) and *B. subtilis* (D). Description: A. Concentration 125 mg/mL, B. Concentration 250 mg/mL, C. Concentration 500 mg/mL, D. Concentration 1000 mg/mL, E. Concentration 2000 mg/mL, F. Positive control, G. Negative control

Table 4. Diameter and wide zone resistor extract ethanol leaf *R. tuberosa* against bacteria *E. coli* and *B. subtilis*

Concentration extract	*Eschericia coli*		*Bacillus subtilis*	
	Diameter(mm)	Area (mm^2)	Diameter(mm)	Area (mm^2)
2000 mg/mL	13.706	147,466	6,696	35,196
1000 mg/mL	7.569	44,972	5,814	26,535
500 mg/mL	6,702	35,259	0	0
250 mg/mL	5,488	23,643	0	0
125 mg/mL	4.253	14,199	0	0
positive control	18,258	261,683	9,586	72.135
Negative control	0	0	0	0

Table 5. Diameter and area of inhibition zone of *R. tuberosa* leaf *n-hexane* extract against *Escherichia coli* and *Bacillus subtilis bacteria*

Concentration extract	*Escherichia coli*		*Bacillus subtilis*	
	Diameter(mm)	Area (mm^2)	Diameter(mm)	Area (mm^2)
2000 mg/mL	9.181	66,154	0	0
1000 mg/mL	0	0	0	0
500 mg/mL	0	0	0	0
250 mg/mL	0	0	0	0
125 mg/mL	0	0	0	0
positive control	18,394	265.596	11.450	102,915
Negative control	0	0	0	0

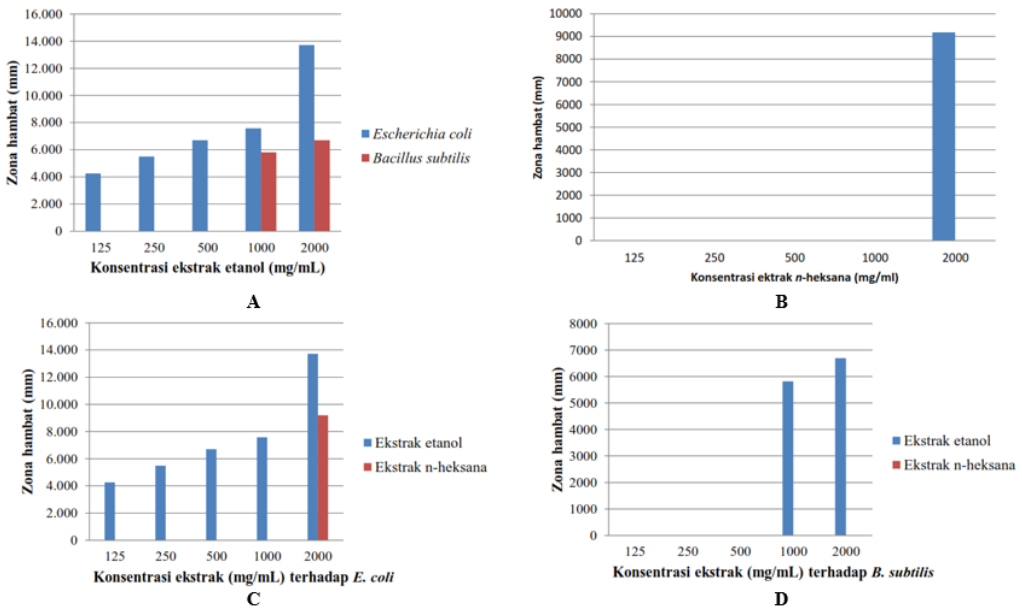

Figure 4. A. Antibacterial activity of *R. tuberosa* leaf ethanol extract against bacteria *E. coli* and *B. subtilis*; B. Antibacterial activity of n-hexane extract *of R. tuberosa* leaves against bacteria *E. coli* and *B. subtilis;* C. Antibacterial activity of *R. tuberosa* leaf extract with ethanol and n-hexane against *E. coli* bacteria; D. Antibacterial activity of *R. tuberosa* leaf extract with ethanol and n-hexane against the bacterium *B. subtilis*.

Table 6. Tukey analysis of inhibition of ethanolic extract of *R. tuberosa leaves* against *Escherichia coli bacteria*.

Variation of extract concentration	Inhibition zone diameter (mm)
125 mg/mL	4.25300 [a]
250 mg/mL	5,48767 [b]
500 mg/mL	6.70233 [c]
1000 mg/mL	7.56967 [c]
2000 mg/mL	13,70567 [d]
positive control	18,25833 [e]

Note: letters a, b, c, d, and e are significant values indicating a significant difference between each treatment

Table 7. Tukey's inhibition of ethanolic extract of *R. tuberosa* leaves against *Bacillus subtilis bacteria*.

Variation of extract concentration	Inhibition zone diameter (mm)
1000 mg/mL	5,81367 [a]
2000 mg/mL	6,69633 [ab]
positive control	9.58600 [b]

Note: letters a, b, c, d, and e are significant values indicating a significant difference between treatments

One-Way ANOVA statistical analysis to see if there are significant differences in the overall treatment. The results of the One-Way Anova test show that = 0.000, so it can be interpreted that there is a significant difference between the five treatment groups above because < 0.05. Because there are differences, it is continued by using the Tukey test, which can be seen in Table 6 and Table 7.

Tukey's statistical analysis in Table 6 shows that the inhibitory power of the ethanol extract of the leaves of *R. tuberosa* at a concentration of 2000 mg/mL is close to the positive control in inhibiting the growth of *E. coli*. Meanwhile, the 125 mg/mL concentration was at least close to the positive control in inhibiting the growth of *E. coli*. Tukey's statistical analysis in Table 7 shows that the inhibitory power of the ethanol extract of the leaves of *R. tuberosa* at a concentration of 2000 mg/mL is close to the positive control in inhibiting the growth of *B. subtilis.* At the same time, the concentration of 1000 mg/mL did not approach the positive control in inhibiting the growth of *B. subtilis.*

In general, the inhibition diameter data in Table 4 and Table 5 showed that the ethanolic extract of *R. tuberosa* leaves had a greater inhibitory power on bacterial growth than the n-hexane extract of *R. tuberosa* leaves. It is due to the presence of secondary metabolites in the test extract. The results of the phytochemical screening test showed that the ethanolic extract of the leaves of *R. tuberosa* contained alkaloids, flavonoids, tannins, saponins, and glycosides. The content of these compounds is more complex than the n-hexane extract of *R. tuberosa* leaves which only contains terpenoid compounds. The more active compounds in the extract, the greater the antibacterial activity. According to Kavitha et al. (2012), an increase in the concentration of the extract causes an increase in the content of active compounds that function as antibacterial so that the antibacterial activity is more significant.

Okeke et al. (2001) and Rahman et al. (2011) stated several secondary metabolites such as glycosides, saponins, tannins, flavonoids, terpenoids, and alkaloids had been reported to have antibacterial activity. Plants synthesize flavone compounds, flavonoids, and flavonols to respond to microbial infections (Ncube et al. 2008). Anyasor et al. (2011) stated that the antibacterial activity of plant extracts might be due to the presence of tannins and flavonoids compounds that bind to the bacterial cell wall and inhibit their biosynthesis.

Flavonoids are synthesized by the plant to respond to infection bacteria and have antibacterial activity in vitro against various pathogenetic. Flavonoids can shape bond complex with extracellular protein and dissolve with Wall cell bacteria, pushing disturbance membrane cell microbes (Moyo et al. 2012). The assembly process of microbial cell walls begins with forming peptide chains that will create peptide cross bridges and combine the glycan chains of peptidoglycan in other chains, causing the cell wall to be perfectly assembled. Suppose there is damage to the cell wall or obstacles in its formation. In that case, lysis of microbial cells can occur so that microbes immediately lose the ability to form colonies and are followed by cell death microbes. Flavonoid compounds inhibit cell wall assembly and incorporate glycan chains not connected to the cell wall peptidoglycan into a weak structure and cause microbial death (Ajizah 2004).

Alkaloids are the largest group of secondary plant substances. Alkaloids have the ability to an antibacterial. The mechanism is by interfering with the peptidoglycan constituent components in bacterial cells so that the cell wall layer is not fully formed and causes cell death (Darsana et al. 2012). The mechanism of bacterial cell wall damage occurs due to alkaloids having primary groups that will come into contact with the amino acids that make up the tetrapeptide which will form cross-bridges in the synthesis of bacterial cell walls. The bond between alkaloids and amino acids results in cross bridges that cause stiffness in the bacterial cell wall not to form. This situation causes bacterial cells to undergo lysis easily, either under physical or osmotic pressure, and causes cell death (Ajizah 2004).

Tannins are polyphenolic compounds that are soluble in water and polar solvents. Tannin compounds can inhibit and kill bacterial growth by reacting with cell membranes. Astringent tannin compounds can induce the formation of complex compounds with bacterial proteins. The bond between astringent compounds and bacterial cell wall proteins causes bacterial proteins to be denatured. When disturbed, proteins that are components of enzymes will interfere with enzyme activity so that the cell walls are formed fragile. As a result, the cell wall becomes weak, and the cell will break or lyse so that the bacteria will die (Akiyama et al., 2001; Moyo et al., 2012).

Saponins work as antibacterial by damaging the cytoplasmic membrane. Damage to the cytoplasmic membrane can reduce the permeability of the cell membrane so that the transport of substances into and out of the cell becomes uncontrolled. Substances inside the cell, such as organic ions, enzymes, amino acids, and nutrients, can leave the cell. If the enzymes out of the cell together with substances such as water and nutrients can cause metabolism to be inhibited, resulting in a decrease in ATP required for cell growth and reproduction, so that bacterial cell growth is inhibited and causes cell death (Hertiani et al. 2003; Rahmawati et al. 2014).

Mechanism terpenoids, as an antibacterial, react with Porin (protein transmembrane) on the membrane outside Wall cell bacteria, shape bond strong polymer, resulting in the damage Porin (Cowan 1999). Terpenes and terpenoids are active in bacteria, fungi, viruses, and protozoa (Enwa et al., 2014).

Chloramphenicol is used as positive control by inhibiting protein synthesis in bacterial cells. Chloramphenicol will bind reversibly to the 50S ribosomal unit, preventing the binding of amino acids to the ribosome. This antibiotic binds specifically to the acceptor (initial binding site of aminoacyl-tRNA) or the peptidyl site, a critical binding site for peptide chain elongation (Katzung 2004).

Besides maximizing the potential of Indonesia's natural resources, which are rich in flora, the development of knowledge about medicinal plants can also minimize the side effects often caused by modern medicines. The

metabolic processes found in plants are primary and secondary metabolism. Primary metabolic processes produce compounds used in daily biosynthesis processes: carbohydrates, proteins, fats, and nucleic acids. On the other hand, secondary metabolic processes produce compounds with certain biological activities, such as alkaloids, terpenoids, flavonoids, tannins, and steroids. Sometimes the compounds contained by a plant of a particular genus are specific. For example, plants from the genus *Papaver*, *Papaver somniferum*, and *Papaver septigerum* produce *morphine* and have calming properties (Hanani 2010).

The function of the first secondary metabolites is to protect plants from microbial attack. For example, plants form phytoalexins, unique compounds synthesized around infected cells. Second, to defend themselves from predators. Third, to fight herbivores' disturbances by creating toxic compounds that cause them to become toxic. Fourth is protecting the environment; for example, anthocyanins are produced to protect plants from UV rays. Fifth, win the competition by producing allelopathic compounds, which are toxic to other plants in the vicinity. Sixth, as an attractant agent, it attracts insects and other herbivores to help disperse seeds. The compound is a pigment that gives the reproductive organs a bright color. It is impossible to contain one kind of secondary metabolite inside one Plants. Certain plants can have several different kinds of therapeutic properties according to the secondary metabolites contained (Hanani 2010).

Ruellia tuberosa leaf extract with solvent ethanol and *n*-hexane

Based on the antibacterial test, it was found that the ethanolic extract of the leaves of *R. tuberosa* gave different antibacterial activity against the two test bacteria compared to the n-hexane extract of the leaves of *R. tuberosa*. Therefore, in determining the minimum inhibitory concentration, the ethanol extract of the leaves of *R. tuberosa* against *E. coli* was used with a test solution concentration of 500; 250; 125; 62.5; 31.25, and 15.625 mg/mL, as well as the concentration of the test solution 1000; 500; 250; 125; 62.5 and 31.25 mg/mL for the ethanolic extract of the leaves of *R. tuberosa* against *B. subtilis.* The minimum inhibitory concentration of *R. tuberosa* leaf ethanol extract against *E. coli* and *B. subtilis* can be seen in Figures 5 and Figure 6.

Table 8. The minimum inhibitory concentration of *R. tuberosa* leaf ethanol extract against *E. coli* and bacteria *B. subtilis*

Concentration extract	OD			% Inhibition	KHM
	I	II	III		
Escherichia coli					
500 mg/mL	0.662	0.580	0.611	99.1	500 mg/mL
250 mg/mL	0.150	0.147	0.148	99	
125 mg/mL	0.283	0.219	0.244	81.1	
62.5 mg/mL	0.202	0.275	0.261	80.9	
31.25 mg/mL	0.630	0.599	0.528	23.4	
15.625 mg/mL	0.606	0.609	0.619	15.4	
Bacillus subtilis					
1000 mg/mL	0.665	0.654	0.654	99	1000 mg/mL
500 mg/mL	0.388	0.380	0.383	82.8	
250 mg/mL	0.545	0.548	0.548	67.8	
125 mg/mL	0.614	0.615	0.615	59.4	
62.5 mg/mL	1.059	1.058	1.061	20.4	
31.25 mg/mL	1,246	1,247	1,248	2.8	

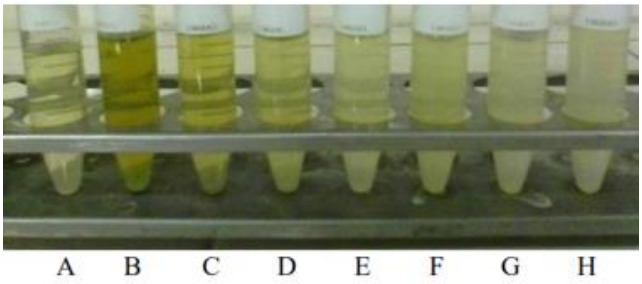

Figure 5. The level of turbidity produced by the ethanol extract of the leaves of *R. tuberosa* against *Escherichia coli bacteria.* A. Control media, B. Concentration 500 mg/mL, C. 250 mg/mL, D. 125 mg/mL, E. 62.5 mg/mL, F. 31.25 mg/mL, G. 15.625 mg/mL, H. Control bacteria

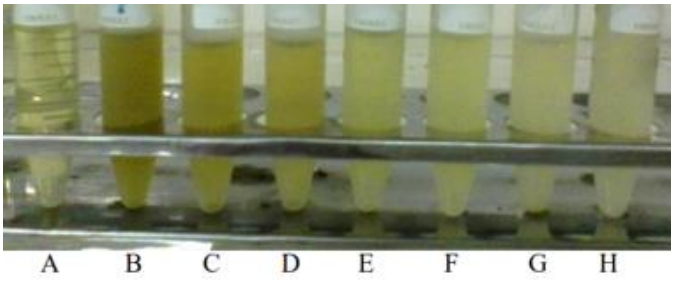

Figure 6. The level of turbidity produced by the ethanol extract of the leaves of *R. tuberosa* against *Bacillus subtilis bacteria*. A. Control media, B. Concentration 500 mg/mL, C. 250 mg/mL, D. 125 mg/mL, E. 62.5 mg/mL, F. 31.25 mg/mL, G. 15.625 mg/mL, H. Control bacteria

From Table 8, it can be seen that the value of the minimum inhibitory concentration for the ethanolic extract of the leaves of *R. tuberosa* against *E. coli* was 500 mg/mL with an inhibition percentage of 99.1%. In comparison, the MIC value for the ethanol extract of the leaves of *R. tuberosa* against *B. subtilis* was 1000 mg/mL with an inhibition percentage of 99%.

The MIC value is expressed as the lowest concentration of extract that can still inhibit the growth of the test bacteria (Pratiwi 2008). The antibacterial MIC value varies depending on the type of bacteria and the antibacterial compounds contained in it. The results of the research showed that the smaller the test concentration, which means the less amount of active substance dissolved in the extract, the lower the ability of the test material to inhibit the growth of a bacterium.

There is a difference in the inhibitory power of the ethanolic extract of the leaves of *R. tuberosa* against the bacteria *E. coli* and *B. subtilis.* The cell walls of Gram-positive bacteria are composed of several layers of peptidoglycan, forming a thick and rigid structure. The thick and rigid cell wall structure makes Gram-positive bacteria resistant to lysis caused by osmotic pressure. On the other hand, Gram-negative bacteria consist of one or very few layers of peptidoglycan in their cell walls. In addition, the cell walls of Gram-negative bacteria contain no teichoic acids but some polysaccharides, making them more susceptible to mechanical and chemical damage (Jawetz et al. 2008).

The glycan chain in *E. coli* is not cross-linked and may be free of part or all tetrapeptide units. Therefore, the cross-linking of peptidoglycan in Gram-positive bacteria is only about 30-70%. In contrast, glycans in Gram-positive bacteria retain all tetrapeptide units completely cross-linked. In addition to variations in cross-linking, variation occurs in the presence of polypeptide-peptidoglycan, polysaccharide, or protein bonds. The glycan chain in *E. coli* and Gram-negative bacteria tend to be straight and surround the cell. *Escherichia coli* contains 106 repeats of tetrapeptide-disaccharide units or sufficient for two or three layers of peptidoglycan. Meanwhile, a Gram-positive cell can contain 20 times the peptidoglycan, sufficient for 40 layers or more (Brock et al. 2006).

The bacteriostatic/bactericidal activity in Figures 7 and 8 showed 500 mg/mL and 250 mg/mL of ethanol extract against *E. coli* bacteria, 1000 mg/mL and 500 mg/mL of ethanol extract against *B. subtilis* were bacteriostatic. It can be seen based on the color produced that does not match the control media, where the color of each extract is cloudy while the color of the controlled media is clear. The cloudy color indicates that there is still bacterial growth. It suggests that the concentration inhibits the growth of *E. coli* bacteria and *B. subtilis.*

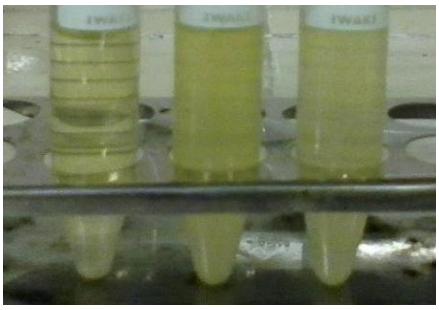

Figure 7. Bacteriostatic/bacteriocidal activity at concentrations of 500 mg/mL and 250 mg/mL ethanolic extract of *R. tuberosa leaves* against *E. coli* bacteria

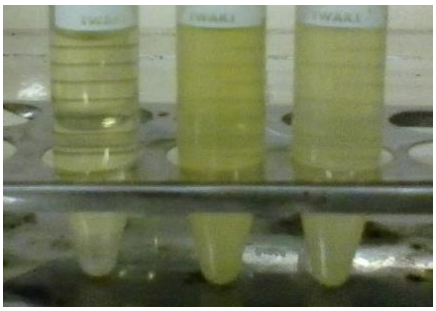

Figure 8. Bacteriostatic/bacteriocidal activity at concentrations of 1000 mg/mL and 500 mg/mL ethanol extract against *B. subtilis* bacteria

Phytochemical screening of *R. tuberosa* leaf extract with ethanol and n-hexane

Phytochemical screening determined the presence of secondary metabolites such as alkaloids, flavonoids, tannins, terpenoids, saponins, and glycosides in the test extract. The obtained ethanol and n-hexane extracts were then tested for phytochemical screening using thin TLC plates (Wagner and Zgainski 1984). Phytochemical screening of ethanol and n-hexane extracts can be seen in Figure 9 A-F.

In the screening of alkaloids, the principle is a precipitation reaction due to ligand replacement. The nitrogen atom, which has a lone pair of electrons on the alkaloids, can replace the iodine ion in the Drgendorf reagent (Marliana et al. 2005). Alkaloids can be found in various parts of plants, but the levels of alkaloids in plant tissues are often less than 1% (Kristanti et al. 2008).

Flavonoids are part of phenolic compounds. Flavonoids have ortho-positioned hydroxy groups that produce yellow fluorescence at UV 366 when reacted with cytroborate. Flavonoids have a type that exists in the free form (aglycone) or is bound as glycosides. Polymethoxy aglycones are non-polar, polyhydroxy aglycones are semi-polar, while flavonoid glycosides are polar because they contain several hydroxyl groups and sugars (Harborne 2006). Therefore, the flavonoid group can be attracted to the polar solvent of ethanol.

The main properties of plant tannins depend on the phenolic group -OH contained in the tannins. The color change indicates tannins after being sprayed with $FeCl_3$, which can react with one of the hydroxyl groups in the tannin compound. Spraying $FeCl_3$ produces a green-black color that shows condensed tannins (Harborne 2006).

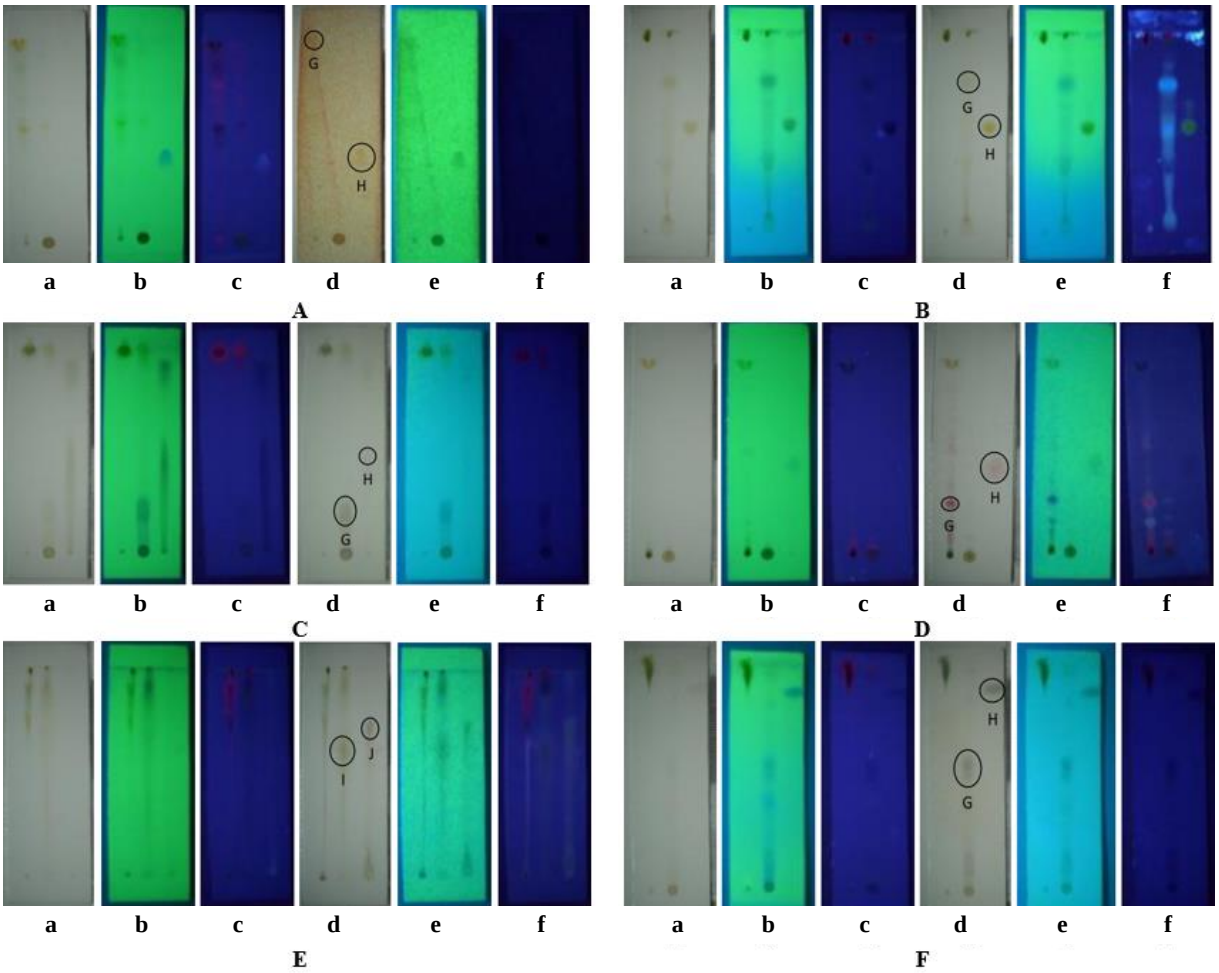

Figure 9. TLC chromatogram on *Ruellia tuberosa* leaves extract. A. The formation of orange spots indicated phytochemical screening of alkaloid compounds after spraying with Dragendorph in visible light; B. Phytochemical screening of flavonoid compounds was indicated by the formation of yellow spots after being sprayed with Sitroborat in visible light; C. Phytochemical screening of tannin compounds was indicated by the formation of dark green-black spots after being sprayed with $FeCl_3$ in visible light; D. Phytochemical screening of terpenoid compounds was indicated by the formation of purplish-red spots after being sprayed with sulfuric acid anisaldehyde in visible light; E. Phytochemical screening of saponin compounds was indicated by the formation of blackish-brown spots after spraying with Lieberman Bourchat in visible light; F. Phytochemical screening of glycoside compounds was indicated by the formation of dark green-black spots after being sprayed with $FeCl_3$ in visible light. Note: a. Before spraying on visible light; b. Before spraying on UV light 254 n; c. Before spraying on UV light 366 nm; d. After being sprayed on visible light; e. After being sprayed on UV light at 254 nm; f. After being sprayed on UV light at 366 nm; G. Rf of ethanolic extract of *R. tuberosa* leaves (A. 0.94, B. 0.75, C. 0.21, D. 0.26, E. 0.6, F. 0.56); H. Rf for comparison (A. quinine 0.42, B. rutin 0.54, C. tannins 0.46, D. tymol 0.42, E. saponins 0.71, F. gallic acid 0.92); I. Rf of saponins ethanol extract of *R. tuberosa* leaves (0.6), J. Rf of saponins as comparison (0.71)

Terpenoids are components commonly found in plants. Most terpenoids contain multiples of five carbon atoms. Terpenoids have a carbon skeleton consisting of two or more C5 units called isoprene (Nagegowda 2010). In the terpenoid test, the compound analysis was based on the ability of these compounds to form colors with concentrated H_2SO_4. The results obtained showed positive results with the formation of purplish-red spots.

The biosynthesis of terpenoids in plants follows the acetic-mevalonic acid pathway. Acetic acid is activated by coenzyme A to form acetyl-CoA and carry out a condensation reaction with other acetyl-CoA to develop acetoacetyl-CoA. The formed acetoacetyl CoA also condenses with other acetyl CoA units, resulting in three combined units of acetyl-CoA, which are further protonated to form mevalonic acid. In the presence of pyrophosphate in mevalonic acid, the release of CO_2 components (decarboxylation) and the release of OPP^- form isopentenyl pyrophosphate (IPP) with its isomer dimethylallyl pyrophosphate (DMAPP) (Dewick 2009).

Saponins are a form of glycosides from polar sapogenins so that ethanol solvents can attract them. Saponins are found mainly in plants. The name saponins are taken from the genus of a plant, namely *Saponaria*, the root of the Caryophyllaceae family can be made into soap (Kristanti et al. 2008).

Phytochemical screening of the ethanolic extract of *R. tuberosa* leaves containing alkaloids, flavonoids, tannins, saponins, and glycosides. Meanwhile, the n-hexane extract of *R. tuberosa* leaves contains terpenoid compounds. The presence of secondary metabolites in each extract was due to the polarity of each solvent that could attract these compounds. Alkaloid compounds, flavonoids, tannins, saponins, and glycosides can generally be attracted by polar solvents such as ethanol (Ncube et al. 2008), while terpenoids are non-polar compounds, which can generally be attracted to non-polar solvents such as *n*-hexane (Tiwari et al. 2011).

Based on literature data on phytochemical and biological investigations of the genus *Ruellia*, the compounds known as secondary metabolites of this genus are flavonoids, alkaloids, terpenoids, and glycosides. They have pharmacological activities, such as antibacterial (Samy et al. 2015). Vasantharaj et al. (2013) stated that the phytochemical tests showed the antibacterial activity of the methanolic extract of *R. tuberosa* leaves due to phytochemical compounds such as alkaloids, terpenoids, tannins, glycosides, and saponins found in these plants.

In conclusion, based on the research results that have been carried out, the following conclusions can be drawn: (i) *R. tuberosa* leaf extract with ethanol and n-hexane as solvents have antibacterial activity against *E. coli* and *B. subtilis.* (ii) Minimum inhibitory concentration of *R. tuberosa* leaf extract with ethanol as a solvent against *E. coli* was 500 mg/mL with an inhibition percentage of 99.1%. In comparison, the minimum inhibitory concentration value for *B. subtilis* was 1000 mg/mL with an inhibition percentage of 99%. (iii) Chemical compounds contained in the leaf extract of *R. tuberosa* with ethanol solvent are alkaloids, flavonoids, tannins, saponins, and glycosides. Meanwhile, the solvent *n*-hexane contains terpenoid compounds.

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The antifungal effect of ethanolic extract of *Aloe vera* leaves on the growth of *Trichophyton rubrum* in vitro

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Abstract. *Salim FS, Djumarga S, Sari Y. 2019. The antifungal effect of ethanolic extract of Aloe vera leaves on the growth of* Trichophyton rubrum *in vitro. Biofarmasi J Nat Prod Biochem 17: 81-85.* This study aims to determine if there is an antifungal effect of the ethanolic extract of *Aloe vera* L. leaves on the growth of *Trichophyton rubrum* in vitro. This study is a quasi-experimental study with a posttest-only-control-group design. Pure *T. rubrum* cultures were obtained from the Microbiology Laboratory, Universitas Setia Budi, Surakarta, Central Java, Indonesia. Sampling was inoculated by simple random sampling with the criteria of a 2-day-old mushroom. Colonies of *T. rubrum* on Sabouraud Dextrose Agar were taken and then diluted with 0.9% NaCl until it reached a turbidity equivalent to 0.5 Mc Farland standard, then smeared evenly on a petri dish containing Sabouraud agar. Samples were divided into 6 groups: negative control in distilled water, extracts with concentrations of 10%, 20%, 40%, 80%, and 100%. Each group consisted of 5 wells. All petri dishes were incubated for 4 days at room temperature. Data in the form of the diameter of the inhibition zone were analyzed using the Kruskal-Wallis test and then continued with post hoc Mann-Whitney analysis. The results showed at least an antifungal effect on the ethanolic extract of *A. vera* leaves on the growth of *T. rubrum* in vitro between the two groups. There were significant differences between the treatment groups except in the concentration group of 10% with 20% and the concentration group of 20% with 40%. There is an antifungal effect on the ethanolic extract of *A. vera* leaves on the growth of *T. rubrum* in vitro, with a concentration of 100% having sensitive results, 80% concentration having intermediate results, and concentrations of 10%, 20%, and 40% having positive results.

Keywords: Antifungal, *Aloe vera, Trichophyton rubrum*

INTRODUCTION

Fungal infection of the skin is a skin disease generally found in Indonesia, a tropical country with hot and humid climates. This infection can affect all levels of society regarding age, economy, and others. Various predispositions supporting this fungus's growth include the lack of public awareness about hygiene and using antibiotics for too long (Harahap 2000; Adiguna 2004).

One of the fungal infections is dermatophytosis, caused by dermatophytes. Dermatophytes are a group of fungi that can digest keratin in the epidermis. Trichophyton is a fungus that causes dermatophytosis in addition to *Microsporum* and *Epidermophyton* (Gandahusada 2003). *Trichophyton* has many species, including *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton schoenleinii*, *Trichophyton tonsurans*, *Trichophyton verrucosum*, and *Trichophyton violaceum* (Patterson 2007; Sari et al. 2012; Jacqueline et al. 2018). The *T. rubrum* is the most common fungus that causes chronic dermatophytosis (Chandra 2006). In one study, Trichophyton fungi were the most common fungi found in samples of skin, hair, finger skin, and nails (Sayuti et al. 2006). The incidence of dermatophytosis of the feet in children in Barcelona, Spain, which is 31.4%, is caused by *T. rubrum* (Gonzalez et al. 2009). Bramono's research (2004) in West Cirebon stated that the most common fungal species causing dermatophytosis was *T. rubrum*, with about 75%.

The use of herbs in alternative medicine is increasing in Indonesia and abroad because it is easy to use and accessible to the public. In addition, the side effects caused by herbal medicines are smaller than chemical drugs. Therefore, herbal medicines are relatively cheaper than chemical drugs (Subroto 2006).

One of the herbal plants is *Aloe vera* L., traditional medicine and cosmetic ingredient. The benefits of *A. vera* can be used to reduce fever, cure hemorrhoids (hemorrhoids) and whooping cough, and accelerate wound healing. In addition, *A. vera* can also be used as an anti-inflammatory, antifungal, cell regeneration, stimulating immunity against cancer, and as nutritional support for PLWHA (People With HIV AIDS) (Widodo and Budiharti 2006).

The *A. vera* contains anthraquinones, especially aloe-emodin and aloin, which can be used as antifungals (Nidiry et al. 2011). Plants other than *A. vera* that can be used as antifungals are *Rheum emodi* Wall. root, which contains anthraquinone derivatives such as rhein, physcion, aloe-emodin, and chrysophanol (Agarwal et al. 2000). Another plant that has a similar effect is the leaves of *Senna Alata* Linn. containing anthraquinone derivatives in the form of rhein and aloe-emodin except for anthraquinone glycoside extract (Wuthi-udomlert et al. 2010).

Various kinds of antifungal tests of *A. vera* extract in vitro have been carried out on several species, such as *Microsporum gypseum*, *Aspergillus flavus*, and *Aspergillus niger* (Sundari and Winarno 2001). Based on the above, the

authors are interested in researching the antifungal effect of *A. vera* leaves on the growth of *T. rubrum* in vitro.

This study aims to determine whether there is an antifungal effect of the ethanolic extract of *A. vera* leaves on the growth of *T. rubrum* in vitro.

MATERIALS AND METHODS

Research sites

Research and measurement of inhibition zones were carried out at the Parasitology Laboratory of the Faculty of Medicine, Universitas Sebelas Maret, Surakarta, and the Microbiology Laboratory, Universitas Setia Budi, Surakarta, Central Java, Indonesia.

Research subject

Pure *T. rubrum* culture was obtained from the Microbiology Laboratory, Universitas Setia Budi, Surakarta, Central Java, Indonesia.

Sampling technique

The inoculated samples can be taken by simple random sampling with the criteria of a-2-day old mushroom. Colonies of *T. rubrum* on Sabouraud Dextrose Agar were taken and then diluted with 0.9% NaCl until it reached turbidity equivalent to 0.5 Mc Farland standards. The physiological solution of 0.9% NaCl acts as a pH buffer so that the cells in the fungus are not damaged due to the decrease in environmental pH. Meanwhile, 0.5 Mc Farland standards standardize the inoculation process (PML Microbiologicals 2001). The *T. rubrum* suspension was taken with a sterile ose and then spread evenly on a petri dish containing Sabouraud agar. The samples were divided into 6 groups, each consisting of 5 wells. The determination of the sample size is based on the Federer formula, namely (Hanafiah 2004):

$$(k - 1)(n - 1) \geq 15$$

Where:
k = number of treatments
n = number of wells per treatment

The number of treatments in this study was 6, consisting of 1 control group and 5 treatment groups. Thus, the minimum value of n is 4. However, in this study, the number of wells was determined to be more than the minimum, namely 5 wells in each group, where the number was still by Federer's formula.

Research tools and materials

Tools: petri dish with a base diameter of 95 mm, a lid diameter of 110 mm, and a height of 18 mm, an ose, a well-making tool with a diameter of 6 mm, an incubator, autoclave, denatured-alcohol lamp, and ruler.

Ingredients: Sabouraud Dextrose Agar, *T. rubrum* culture obtained from the Microbiology Laboratory of Universitas Setia Budi, Surakarta, Central Java, Indonesia ethanol extract of *A. vera* leaf from LPPT (Integrated Research and Testing Laboratory) Universitas Gajah Mada. Yogyakarta, Indonesia, antibiotic Kalmicetine capsule containing 250 mg chloramphenicol produced by Kalbe Pharma.

Antibiotic preparation of chloramphenicol (Bridson 2006)

The antibiotic dose of chloramphenicol in SDA solution was 0.4 g/L, and the dose of 0.9% NaCl solvent in 250 mg chloramphenicol was 10 mL. One capsule of 250 mg chloramphenicol is dissolved in 10 mL of 0.9% NaCl. A 1.6 mL mixture of chloramphenicol and 0.9% NaCl was taken as the antibiotic dose in the sabouraud agar solution.

Preparation of agar plates (Bridson 2006)

The fungus *T. rubrum* was reared on a slanted Sabouraud Dextrose Agar (SDA). First, 100 mL of distilled water was added to 6.5 grams of SDA powder. It is then heated and stirred until it becomes homogeneous. A total of 1.6 mL of a mixture of chloramphenicol and 0.9% NaCl that had been prepared was dissolved in a solution of sabouraud agar and then stirred until evenly dissolved. SDA tools and media were sterilized in an autoclave at 121ºC for 15 minutes. SDA liquid medium was poured into a petri dish with a thickness of 20 mL. Let it solidify.

Preparation of mushroom suspension

The *T. rubrum* culture was taken using a sterile ose and then put into a 0.9% NaCl solution, shaken, and stirred until it reached a turbidity equivalent to 0.5 Mc Farland standard. The suspension of *T. rubrum*, shaken and stirred, was immediately inoculated on the plate to solidify. The suspension was shaken and stirred just before the inoculation of each petri dish to prevent precipitation of the suspension. Petri dishes that the suspension has inoculated are then incubated at room temperature for 4 days.

Preparation of *A. vera* ethanol extract

A total of 5,926 grams of *A. vera* leaves are split to take the gel. Then, the gel was dried in a drying cabinet at 45ºC for 48 hours. The dried form of *A. vera* leaves was obtained, weighing 59 grams. The dried form of *A. vera* leaves was put into a pollinator machine to obtain a powder form of *A. vera* leaf weighing 58.410 grams. The *A. vera* leaf dry powder was macerated with 70% ethanol solvent using a homogenizer. Then shaken for 30 minutes and precipitated for 24 hours. The results were then filtered using a Buchner funnel to obtain the first pulp and filtrate. The first dregs were dissolved with 70% ethanol solvent, shaken for 30 minutes, and allowed to stand for 24 hours. The results were then filtered using a Buchner funnel to obtain the second dregs and filtrate. The second dregs were redissolved with 70% ethanol, shaken again for 30 minutes, and allowed to stand for 24 hours. The results were then filtered using a Buchner funnel to obtain the third dregs and filtrate. The three filtrates were combined and evaporated using a vacuum rotary evaporator with a

water bath heating at 70ºC to obtain a thick extract weighing 30.950 grams. The thick extract was poured into a porcelain cup and then heated in a water bath at 70ºC. The extract was diluted with distilled water to reach 100% (no dilution), 80%, 40%, 20%, and 10%, with a volume of 3 mL for each concentration.

Treatment

Six wells with a diameter of 6 mm were made in 5 inoculated petri dishes. First, the well was added 0.5 mL of the ethanolic extract of *A. vera* and distilled water. Then it was incubated at room temperature for 4 days. The clear area around the well was measured with a ruler. Measurements were made at the bottom of the petri dish by calculating the average diameter of the smallest and largest inhibition zones. The interpretation of the diameter of the sensitivity zone is as follows (Hoffman and Michael 2001): (i) 19 mm or more: sensitive, (ii) 13-18 mm: intermediate, and (iii) 12 mm: resistant. Data were tabulated, averaged, and evaluated.

Data analysis

The analysis was carried out statistically using the one-way ANOVA test with a significance of p = 0.05 and continued with the Tukey Honestly Significant Difference (HSD) test. The ANOVA test was carried out to determine if there were differences in the inhibition zones between the 5 groups by comparing the average inhibition zones of the groups. The Tukey HSD test was used to determine if there was any significance between groups in inhibiting the growth of *T. rubrum*.

The requirements for the ANOVA test are the sample must consist of more than two groups, the data distribution must be normal, and the data variance must be homogeneous. The second condition is if the significance of the p-value is <0.05, then the ANOVA test is continued with post hoc analysis. Finally, suppose one of the ANOVA test conditions is not met. In that case, it can be continued with nonparametric tests such as the Kruskal-Wallis test and then with post hoc analysis of the Mann-Whitney test (Priyatno 2009).

RESULT AND DISCUSSION

After researching the antifungal effect of ethanolic extract of *A. vera* on the growth of *T. rubrum* in vitro, data was collected on the 4th day of incubation.

The diameter of the inhibition zone for each group can be seen in Table 1. In the distilled water group, 0 mm was obtained in the five replicates, indicating that the distilled water used as a diluent extract did not affect the growth of fungi. The average diameter of the inhibition zones in the ethanol extract of *A. vera* at concentrations of 10%, 20%, 40%, 80%, and 100%, respectively, was 8.8 mm, 10.9 mm, 11.6 mm, 18.6 mm, and 22.8 mm.

Based on Table 1, Figure 1 can depict the average of various administration treatments of a certain concentration of ethanol extract with the diameter of the *T. rubrum* inhibition zone in each treatment group.

Figure 1 shows the difference in the average diameter of the inhibition zone for each extract concentration with distilled water as a negative control. It can be seen in Figure 1 that the diameter of the inhibition zone increased with the increase in the concentration of *A. vera* ethanol extract.

Data analysis

The research data regarding the antifungal activity test of *A. vera* ethanol extract on the growth of *T. rubrum* in vitro were analyzed by a one-way ANOVA test, which was then followed by a post hoc Tukey HSD test if the ANOVA test had normal, homogeneous data distribution, and had p<0.05. The data was processed using Statistical Product and Service Solution (SPSS) 17.0 for Windows software. If one of the ANOVA test conditions is not met, proceed with the Kruskal-Wallis nonparametric test followed by the Mann-Whitney post hoc test.

The test of normality using the Shapiro-Wilk analytical method showed that the inhibition zone data had an abnormal data distribution. Thus, the ANOVA test could not be performed because the data were not normally distributed. Then it is continued by conducting nonparametric statistical tests such as Kruskal-Wallis.

In the Kruskal-Wallis test, p = 0.00 (p <0.05). Thus, it can be concluded that there is at least a difference in the antifungal effect between the two groups of extract concentrations. Furthermore, post hoc analysis was conducted to determine the significance between treatment groups. The test was the post hoc Mann-Whitney test.

Table 1. The measurement results in the diameter of the inhibition zone of *Trichophyton rubrum*

Repetition	Aquadest (Distilled Water)	Diameter of inhibition zone *A. vera* ethanol extract				
		10%	20%	40%	80%	100%
I	0	9	12	11	18	22
II	0	7.5	9.5	9.5	18.5	23
III	0	8	9	12	17	24.5
IV	0	10	12	11.5	19	22.5
V	0	9.5	12	14	20.5	22
Average	0	8.8	10.9	11.6	18.6	22.8

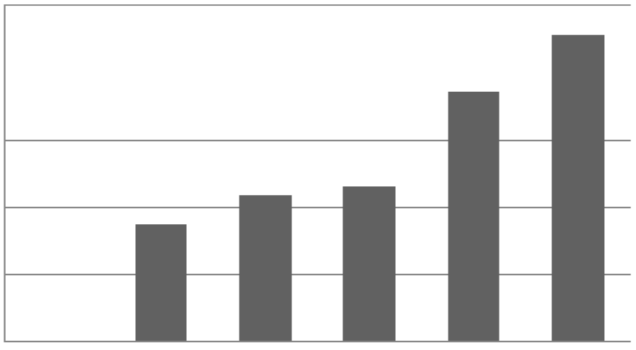

Figure 1. The average diameter of the inhibition zone (mm) in each treatment group

A post hoc Mann-Whitney analysis test on the antifungal effect of *A. vera* ethanol extract on the growth of *T. rubrum* in vitro was used to determine the significance between treatment groups. The results of the Mann-Whitney test showed that the negative control group in the form of distilled water had significant differences from other treatment groups, namely extracts with concentrations of 10%, 20%, 40%, 80%, and 100%. It can also be seen in the results of the Mann-Whitney test that the differences between the treatment groups had significant results ($p<0.05$) except between the 10% extract group with the 20% extract group and the 20% extract group with the 40% extract group which did not have significant results ($p>0.05$).

Discussion

The antifungal sensitivity test is a test to determine the effectiveness of antifungal agents in inhibiting the growth of fungi that infect humans. Fungal samples in patients were cultured in culture and then tested for sensitivity using various antifungal agents. Interpretation of the results can be sensitive, intermediate, or resistant. Thus, the doctor can determine the sensitive antifungal agent suitable for the patient.

The *A. vera* has antifungal content in anthraquinone with the main substances aloe-emodin and aloin (Nidiry et al. 2011). In an in vitro study, *T. rubrum* fungus, given with ethanol extract of *A. vera* leaves in different concentrations, had different inhibitory powers. The Mann-Whitney test analysis can be carried out to find out the average diameter of the inhibition zone between groups. Antifungal measurements can be made by measuring the diameter of the clear zone around the well.

Extract dilution into various concentrations using distilled water. It is expected that the distilled water in the ethanolic extract of *A. vera* leaves will not affect the inhibition of the fungus *T. rubrum* because there is no inhibition zone around the wells given with aquadest. Thus, distilled water did not interfere with extracts with various concentrations and had no antifungal effect.

Analysis of the Mann-Whitney test results yielded data that the comparison between groups had significant differences except between extracts with a concentration of 10% and 20% and extracts with a concentration of 20% and 40%. From the results of the study, it was proven that the ethanolic extract of *A. vera* had an antifungal effect. The *A. vera*, which has anthraquinones, especially aloe-emodin and aloin, has been shown to have antifungal properties, as stated by Nidiry et al. (2011). However, according to Phongpaichit et al. (2004), the mechanism of action of anthraquinones is still unclear. Possibly, there is a leak in the cell wall or a change in cell permeability, which eventually causes the fungal cell to lose its cytoplasm.

According to Hoffman and Michael (2001), the interpretation of the diameter of the sensitivity zone having sensitive results is the inhibition zone with a diameter of 19 mm or more. The results showed that only the concentration of 100% had an average of more than 19 mm, which was 22.8 mm. Thus, it can be said that the ethanol extract of *A. vera* leaves with a concentration of 100% effectively inhibited the growth of *T. rubrum* as a cause of dermatophytosis.

The 80% concentration has an average of 18.6 mm. It means that it is an intermediate category based on the opinion of Hoffman and Michael (2001). It indicates that the ethanol extract of *A. vera* leaves with a concentration of 80% is effective in inhibiting the growth of *T. rubrum* as a cause of dermatophytosis on the condition that the dose is given higher or the dose is given more often.

Ethanol extract with concentrations of 10%, 20%, and 40% had an average diameter of inhibition zone of less than 12 mm, which means that the three concentrations were resistant to *T. rubrum*. Thus, the ethanol extracts at concentrations of 10%, 20%, and 40%, although having antifungal effects, were not effective in inhibiting the growth of *T. rubrum*. The results of this study support the research conducted by Arunkumar and Muthuselvam (2009), which stated that the ethanolic extract of *A. vera* has an antifungal effect against *A. niger* and *A. flavus*. Therefore, in this study, it can also be concluded that the ethanol extract of *A. vera* with a concentration of 100% has an antifungal effect on the growth of *T. rubrum*.

Based on the research that has been done, it can be seen that there is an antifungal effect on the ethanolic extract of *A. vera* leaves on the growth of *T. rubrum* in vitro with a concentration of 100% having sensitive results, 80% concentration having intermediate results. On the other hand, a concentration of 10%, 20%, and 40% had resistant results.

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Antipyretic and anti-inflammatory effect of dichloromethane-methanolic extracts of *Ximenia americana* leaves and barks in rats and mice models

GAICHU DANIEL MUTHEE[1], MATHEW NGUGI[2,♥], DAVID MBURU[2]
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Abstract. *Muthee GD, Ngugi M, Mburu D. 2018. Antipyretic and anti-inflammatory effect of dichloromethane-methanolic extracts of Ximenia americana leaves and barks in rats and mice models. Biofarmasi J Nat Prod Biochem 17: 86-96.* Extremely high body temperatures lead to the destruction of cells in the body, while excessive inflammation damages the tissues and organs of the body, requiring treatment of fever and inflammation. *Ximenia americana* L. is used in Africa as a traditional medicine to treat various ailments such as pain, helminthiasis, inflammation, fever, wounds, diarrhea, and poisoning. This study tested the antipyretic and anti-inflammatory activity of methanolic dichloromethane of *X. americana* leaf and stem bark extracts in rats and mice, respectively. The plant material was collected in Mbeere in Embu County, Kenya. The active ingredients were extracted with methanol and dichloromethane in a 1:1 ratio. Two- or three-month-old Wister rats were used to test antipyretic activities, while five- to six-week-old Swiss albino mice were used to test anti-inflammatory activities. The animals were divided into six groups of five each; negative control, reference control, normal control, and three experimental groups (50, 100, and 150 mg/kg). Pyrexia was experimentally induced with turpentine, while inflammation was induced with carrageenan. The experimental groups were treated with predetermined doses of prepared extracts, and one-way ANOVA analyzed the data. The antipyretic and anti-inflammatory effects in rats and mice were compared with aspirin (100 mg/kg) and diclofenac (15 mg/kg) as conventional standard drugs. Leaf extracts reduced rectal temperature from 0.45% to 2.11%, while stem bark extracts reduced rectal temperature from 0.71% to 2.13%. Aspirin reduced the rectal temperature by 0.74% to 1.67%. In anti-inflammatory studies, leaf extract reduced inflammation by 0.91% to 16.90%, while stem bark extract reduced inflammation by 5.84% to 29.00%. Diclofenac reduced inflammation from 1.32% to 29.60%. Qualitative phytochemical screening identified the presence of alkaloids, flavonoids, saponins, cardiac glycosides, phenols, and terpenoids. Flavonoids, alkaloids, and saponins have been associated with antipyretic and anti-inflammatory effects. The study found that *X. americana*'s DCM methanolic extract effectively treated fever and inflammation. In conclusion, taken together, *X. americana* can be explored as a potential biological source for generating a readily available herbal formulation effective in treating fever and inflammation.

Keywords: Antipyretic, anti-inflammatory, dichloromethane-methanolic extracts, *Ximenia americana*

INTRODUCTION

Fever also called pyrexia, or febrile reaction, is defined as a higher than normal body temperature due to an increase in the thermoregulatory set point (Axelrod and Diringer 2008). However, there is no agreed upper limit for the normal temperature at sources using values between 37.5 and 38.3°C (99.5 and 100.9°F) (Laupland 2009). Fever is generated from the release and conversion of arachidonic acid from cellular lipid membranes to prostaglandin E2 through the action of cyclooxygenase enzymes COX-1 and COX-2 (Purssell and While 2013).

Conversely, inflammation is part of the complex biological response of vascular tissue to noxious stimuli such as pathogens and damaged or irritating cells (Ferrero-Miliani et al. 2007). Inflammation is a protective immune-vascular response involving immune cells, blood vessels, and molecular mediators (Bairy et al. 2015). The classic signs of acute inflammation are pain, warmth, redness, swelling, and loss of function (Abbas and Lichtman 2009). Certain diseases, such as rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, retinitis, multiple sclerosis, psoriasis, and atherosclerosis, do not seem to improve. A chronic inflammatory disease may last a lifetime (Neville et al. 2004).

The main effect of antipyretics is their ability to inhibit the enzyme cyclooxygenase (COX) and disrupt the synthesis of inflammatory prostaglandins (Weissmann 1991). However, recent studies have revealed many serious side effects of conventional medications independent of COX inhibition (Biren and Avinash 2010). Non-steroidal anti-inflammatory drugs are used worldwide to treat inflammation (Narsinghani and Sharma 2014). Synthetic drug use typically causes many adverse effects (Parveen et al. 2014). Medicines of natural origin are an important source for the treatment of many diseases in the world (Pandima et al. 2003). As an alternative to chemotherapy, natural active ingredients with high efficacy and few side effects would be desirable (Rodrigo et al. 2013). Today, nearly 80% of the world's population uses herbal medicines for primary health care because of their efficacy, availability (Snyderman and Weil 2002), and minimal side

effects. These plants have been used long ago as a source of antipyretic and anti-inflammatory effects, the discovery of antipyretic and anti-inflammatory chemicals has meant that they have long been ignored. Still, fortunately, many are returning to herbal remedies for various reasons. Graz and Malebo 2011).

Ximenia americana L. is used in different countries as an alternative medicine to treat various human diseases (Urso et al. 2013; Ekandjo et al.2018). In Burkina Faso, the fruit is used as a remedy for constipation and as a natural source of astringent and tonic substances (Lamien-Meda et al. 2008). Mali root treats skin diseases (Diallo et al. 2002; Grønhaug et al. 2008). In Zimbabwe, a decoction of leafy twigs is used to treat colds and coughs and as a laxative (Okigbo et al. 2009). Nigeria uses the same preparation as mouthwash to relieve toothache (Dalziel 1937). In Kenya, the communities such as Mbeere and Tharaka use the leaves, bark, and roots more diversely for human food, medicine, and animal feed (Feyssa et al. 2012).

However, in addition to the popular *X. americana*, Soro et al. (2015) data on the antipyretic and anti-inflammatory effects of aqueous plant extracts, there are no published data on the antipyretic and anti-inflammatory effects of the organic extracts of *X. americana*. In this context, the study aimed to bio-screen methanolic stem bark and leaf extracts of X DCM. Antipyretic and anti-inflammatory effects of *X. americana* in laboratory rats and mice are the first step in developing herbal antipyretic and anti-inflammatory drugs.

MATERIALS AND METHODS

Collection and preparation of plant materials

Stem bark and leaves of *X. americana* were collected from Siakago in Mbeere North Sub-district, Embu County, Kenya, with the help of local herbalists. Information collected includes common names, preparation methods, potency seasonality, and plant parts used. The materials were carefully selected, cleaned, and transported in plastic bags to Kenyatta University, Biochemistry and Biotechnology Laboratories, then dried and ground. Materials were collected using acceptable biological conservation methods. The plant material was provided to a registered taxonomist for botanical authentication, and a copy of the specimen was deposited in the Kenyatta University Herbarium, Nairobi, Kenya. The leaves and stem bark of *X. americana* were cut into small pieces and air-dried at room temperature until completely dry. They were then ground into a fine homogeneous powder using an electric grinder, followed by sieving through a mesh sieve.

Extraction

For each sample, 200 grams of leaf and stem bark powder were separately soaked in a cold 1: 1 mixture of methanol: DCM and stirred for six hours to extract the compounds. The sequential extract was filtered, and the filtrate was concentrated under reduced pressure and vacuum using a rotary evaporator (Buchi R110). The concentrate was placed in an airtight container for biological analysis studies and stored at -4°C.

Laboratory animals

Male and female Wister rats aged 2-3 months weighing 140-180 g and both sexes of Swiss albino mice aged 5-6 weeks weighing 15-35 g were used in the antipyretic test (Khan et al. 2013). Animal breeding colonies were obtained and housed in the breeding and testing facility of the Department of Biochemistry and Biotechnology, Kenya University. Animals were housed in cages and kept at room temperature. They were fed standard rodent pellets and watered ad libitum (Kirkham et al. 2002). Ethical guidelines and procedures for handling laboratory animals (Olfert et al. 1993) were followed throughout the study.

Experimental design

Determination of antipyretic activities

Thirty animals were used and divided into 6 groups of 5 rats, each treated as follows. Group, I (normal control) was normal and received DMSO. Another group was induced with fever (negative control) and administered DMSO. The third group was induced with fever (reference control) and received aspirin (reference drug). Finally, groups IV, V, and VI were experimental groups that induced inflammation and received test doses of 50, 100, and 150 mg/kg body weight, respectively (Table 1).

Turpentine solution (20%) was used as an antipyretic. Before fever induction, rats were weighed, and rectal temperature was recorded by inserting the thermistor probe of a well-lubricated digital thermometer (model YB-009). Three (3) cm in the rectum (Grover 1990). The digital thermometer is calibrated for a mercury thermometer. After baseline basal rectal temperature was measured and recorded, rats were injected with 20% turpentine at a dose of 20 mL/kg body weight to induce fever and left for 1 hour. The degree of fever induced by an intraperitoneal injection of turpentine 1 hour later was defined as a 100% febrile response. Therefore, only rats with a body temperature increase of 0.8°C were considered febrile and used in the study. All extracts are soluble in DMSO.

The temperature was recorded every hour until the fourth hour after the turpentine injection. Rectal temperatures before and after treatment were compared, and percentage changes in rectal temperature were calculated using the following formula (Hukkeri et al. 2006; Ray et al. 2006):

$$\frac{B - Cn}{B} \times 100$$

Where, B: Rectal temperature at 1 h after turpentine administration; C_n: Rectal temperature after drug administration

Determination of anti-inflammatory activities

Those experimental animals were divided into six groups, with five mice per group, then treated as follows: Group I (normal control group) was normal and administered in DMSO. On the Group II (negative control) inflammation was induced and received DMSO. The third

group (reference control) was induced with inflammation and received diclofenac (reference drug). Finally, groups IV, V, and VI are test groups that induce inflammation and receive 50 mg/kg body weight, 100 mg/kg body weight, and 150 mg/kg body weight, respectively (Table 2).

Paw inflammation was induced by injecting carrageenan (0.1 mL, 1% w/v in saline) into the subplantar tissue of the right hind paw (Winter et al. 1962). Plant extracts and DMSO were administered intraperitoneally one hour after induction of inflammation. Edema was measured immediately before carrageenan injection and compared with the volume of the same paw after carrageenan injection. Linear paw circumferences were measured at hourly intervals of 4 hours (Bamgbose and Noamesi 1981) and compared using the formula:

$$E_1 = \frac{t_1 - t_0}{t_0} X\ 100$$

Where: t_0: the initial paw circumference,t_1: the final paw circumference.

Qualitative phytochemical screening

A qualitative phytochemical screen was performed on the obtained extracts to identify the presence of selected phytochemicals using the protocols described by Harbone (1998) and Kotake (2000). In addition, the following secondary metabolites were tested: alkaloids, tannins, steroids, saponins, cardiac glycosides, phenols, and terpenoids.

Table 1. The treatment protocol to evaluate of antipyretic activities of DCM-Methanolic leaf and stem bark extracts *Ximenia americana* in rats

Group	Status	Treatment
I	Normal control	DMSO
II	Negative control	Turpentine (20%) + DMSO (10%)
III	Positive control	Turpentine (20%) +100 mg/kg Aspirin
IV	Experimental group A	Turpentine (20%) +50 mg/kg extract
V	Experimental group B	Turpentine (20%) +100 mg/kg extract
VI	Experimental group C	Turpentine (20%) +150 mg/kg extract

Table 2. The treatment protocol to evaluate of anti-inflammatory activities of DCM-Methanolic leaf and stem bark extracts *Ximenia americana* in mice

Group	Status	Treatment
I	Normal control	DMSO
II	Negative control	Carrageenan 100 µg + DMSO (10%)
III	Positive control	Carrageenan 100 µg + 15 mg/kg diclofenac
IV	Experimental group A	Carrageenan 100 µg + 50 mg/kg extract
V	Experimental group B	Carrageenan 100 µg + 100 mg/kg extract
VI	Experimental group C	Carrageenan 100 µg + 150 mg/kg extract

Alkaloids

The extracts were subjected to qualitative phytochemical screening for the presence of selected phytochemicals using the protocol described by Harbone (1998) and Kotake (2000). In addition, the following secondary metabolites were examined: alkaloids, tannins, steroids, saponins, cardiac glycosides, phenols, and terpenoids.

Flavonoids (Sodium hydroxide test)

Test the extracts for flavonoids by mixing 2 mL of each extract with 2 mL of 5 M sodium hydroxide (NaOH). A strong golden yellow precipitate indicates a positive result.

Steroids

A sum of 0.5 g of each extract was dissolved in 2 mL of chloroform. Then, approximately 3 mL of 2M H_2SO_4 was gently added to the sides of the tube to form a layer. A reddish brown color on the skin indicates the presence of a steroid ring.

Saponins (Froth test)

In the saponins test, 2 mL of the plant extract was mixed with 2 mL sodium bicarbonate solution and shaken vigorously. The extract was then left to stand for 15-20 minutes. The formation of foam indicated the presence of saponins.

Cardiac glycosides (Keller-Kilian test)

To test cardiac glycosides, dissolve 0.5 g of the extract in 2 mL of glacial acetic acid containing 2 drops of 10% ferric chloride ($FeCl_3$). One (1) mL of concentrated sulfuric acid was added to it. A brown, purple, or green ring at the interface indicates the presence of a deoxysugar with a cardenolide character. A purple ring may appear below the brown ring, while a green ring may form in the acetate layer directly above the brown ring, gradually spreading through the layer.

Phenols

The extracts were tested for phenolics by adding 1 mL of ferric chloride solution to 2 mL of each extract. The formation of a blue to the green color indicated the presence of phenols.

Terpenoids (Salkowski test)

Add each 0.5 g of the extract to 1 mL of ethyl acetate/petroleum ether and mix with 2 mL of chloroform. Then carefully add 3 mL of 2M sulfuric acid (H_2SO_4) to form a layer. The interface is red-brown, indicating a positive result for terpenoids.

Data management and statistical analysis

Experimental data on changes in rectal temperature and right buttock circumference were obtained from all animals in different groups, recorded, and compiled in a spreadsheet. Descriptive statistics were performed on the results, expressed as mean ± standard error of analysis (SEM), and the hypothesis was tested at $p \leq 0.05$ to be considered significant.

Differences between groups were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey's test for pairwise separation and comparison of means. In addition, an unpaired Student's t-test was performed to compare the mean activity of leaf and root bark extracts. Analyze data using Minitab Statistical software version 17.

RESULTS AND DISCUSSION

The antipyretic activity of DCM-methanolic leaf and stem bark extracts of *Ximenia americana* in rats

The treatment with DCM-methanolic leaf extracts of *X. americana* on rats showed some antipyretic activity against turpentine fever, as evidenced by the reduction in rectal temperature (Table 3; Figure 1). In the first hour after treatment, *X. americana*'s DCM methanolic leaf extracts at doses of 50,100 and 150 mg/kg body weight elevated rectal temperatures by 0.98%, 0.93%, and 1.27%, respectively (Figure 1). In addition, the antipyretic activities of the extract at doses of 50, 100, and 150 mg/kg were statistically significant ($p<0.05$; Table 3) compared to those of the normal and negative control groups. However, the antipyretic effect of the extract was not statistically significant at all doses compared to the positive control group ($p<0.05$; Table 3).

In the second hour, the DCM-methanolic leaf extracts of *X. americana* at doses of 50, 100, and 150 mg/kg showed temperatures below 1.37%, 1.56%, and 1.76%, respectively (Figure 1). The antipyretic efficacy of the extracts was statistically significant ($p<0.05$; Table 3) compared to the normal and negative control groups but not statistically significant compared to the positive group ($p>0.05$; Table 3).

By the third hour, *X. americana* reduced the elevated rectal temperature by 0.9%, 1.45%, and 2.05% at all doses (50, 100, and 150 mg/kg body weight, respectively) (Figure 1). At this time, rats treated with doses of 100 and 150 mg/kg body weight of the extract showed antipyretic activity comparable to the positive control group ($p>0.05$; Table 3). In addition, the antipyretic activity of the extract was statistically significant ($p<0.05$; Table 3) at all doses (50, 100, and 150 mg/kg body weight) compared to normal and negative control groups.

After 4 hours of treatment, all doses of *X. americana* leaf extract (50, 100, and 150 mg/kg body weight) were found to reduce turpentine-induced fever by 0.45%, 1.19%, and 2.11%, respectively (Figure 1). The antipyretic activity of the extract at doses of 100 and 150 mg/kg body weight was comparable to the positive control with aspirin as a standard drug ($p>0.05$; Table 3) and was statistically significant compared to the normal and negative controls. ($p<0.05$) 0.05, Table 3). Moreover, the antipyretic effect of the extract at 50 mg/kg body weight was not significantly different from normal and negative control ($p>0.05$; Table 3).

Similarly, the DCM-methanol stem bark extract of B. americana had antipyretic activity against turpentine-induced fever in rats (Table 4; Figure 2). In the first hour of the study, *X. americana* DCM-methanol stem bark extract at doses of 50, 100, and 150 mg/kg body weight reduced rectal temperature in rats by 0.91%, 0.71%, and 0.88%, respectively. At the same time, aspirin reduced the rectal temperature by 0.74% (Figure 2). The activity of the extract at three dose levels (50, 100, and 150 mg/kg body weight) was comparable to that of aspirin ($p= 0.05$; Table 4). At a dose level of 100 mg/kg body weight, the activity of the extract was not significantly different from the normal control group ($p= 0.05$; Table 4). However, at 50 and 150 mg/kg body weight, the effect of the extract was significantly different from the normal and negative controls ($p<0.05$; Table 4).

In the second hour, methanolic extracts of stem bark of *X. americana* DCM at doses of 50, 100, and 150 mg/kg body weight reduced rectal temperature in rats by 1.49%, 1.54%, and 1.87%, respectively, depending on the dose. (Figure 2). The antipyretic efficacy of the extract was comparable to that of the positive control group at all three doses ($p>0.05$; Table 4; Figure 3).

In the third hour, *X. americana* stem bark extracts with methanolic DCM at 50, 100, and 150 mg/kg body weight significantly reduced the rectal temperature by 1.44%, 1.50%, and 2.13%, respectively (Figure 2). In addition, the groups treated with DCM-methanolic *X. americana* stem bark extract at doses of 50, 100, and 150 mg/kg body weight showed slightly different antipyretic activity than the positive control group ($p= 0,05$; Table 4).

During the fourth hour, stem bark extracts of DCM-methanolic *X. americana* at doses of 50, 100, and 150 mg/kg body weight reduced the rectal temperature by 1.28%, 1.28%, and 2.02%, respectively (Figure 2). Similarly, at this time, groups treated with DCM-methanolic *X. americana* stem bark extract at doses of 50, 100, and 150 mg/kg body weight showed slightly different antipyretic activity. Others, as well as the positive control group ($p>0.05$; Table 4).

Furthermore, there was no significant difference in the antipyretic effect at the three dosage levels of 50, 100, and 150 mg/kg body weight during the multi-hour test of the leaf and stem bark extract of *X. americana*. During the 4-hour experimental period, the level of significance was as follows: at a dose of 50 mg/kg body weight ($p= 0.872$, 0.821, 0.307, and 0.176, respectively), at 100 mg/kg body weight ($p= 0.084$, 0.549, 0.769, and 0.501) and at 150 mg/kg body weight ($p= 0.205$, 0.608, 0.768, and 0.747, respectively).

Evaluation of the anti-inflammatory activity of DCM-Methanolic leaf andstem bark extracts in rats of *Ximenia americana*

The treatment of rats with DCM-methanolic leaf extract of *X. americana* at the 50,100 and 150 mg/kg body weight reduced carrageenan-induced inflammation in mice, which was observed by the reduction in the circumference of the hind leg.

In the first hour of the trial period, DCM-Methanol *X. americana* leaf extract at the three dose levels of 50,100 and 150 mg/kg body weight reduced the leg circumference by 0.91%, 28%, and 2.30%, respectively (Figure 4). At this

time, the anti-inflammatory activity of the DCM methanolic extract of leaves of *X. americana* at the three doses was not significantly different from the positive control group (p>0.05; Table 5). However, due to the activity of the extract at the dose of 100 and 150 mg/kg body weight was significantly different compared to the normal and negative control groups (p <0.05; Table 5).

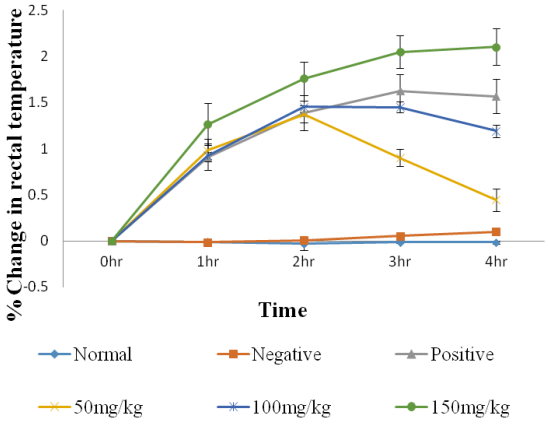

Figure 1. The percentages change in rectal temperature by DCM-Methanolic leaf extract of *X. americana* on turpentine-induced pyretic rats

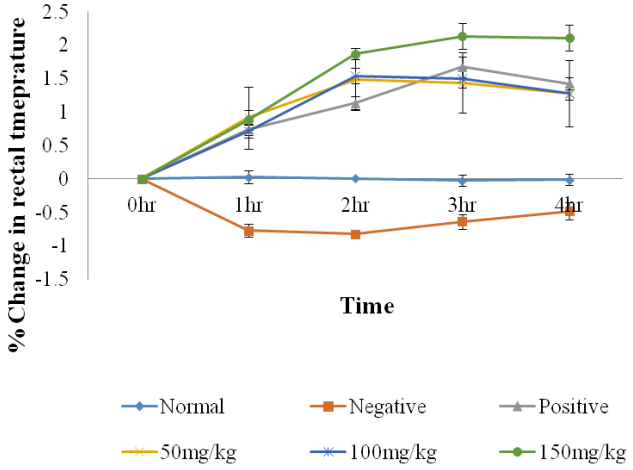

Figure 2. The percentages change in rectal temperature by DCM-Methanolic stem bark extract of *Ximenia americana* on turpentine-induced pyretic rats

Table 3. The effects of intraperitoneal administration of DCM-Methanolic leaf extracts of *X. americana* onturpentine-induced pyrexia in rats

Group	Treatment	Percent change in rectal temperature (°C) after drug administration				
		0h	1h	2h	3h	4h
Normal control	DMSO (10%)	100±0.00	100.01 ± 0.02^{a}	100.03 ± 0.07^{a}	100.02 ± 0.01^{a}	100.02 ± 0.01^{a}
Negative control	Turpentine	100±0.00	100.02 ± 0.02^{a}	99.995 ± 0.02^{a}	99.947 ± 0.01^{a}	99.900 ± 0.02^{a}
Positive control	Turpentine + Aspirin (100mg/Kg b.w)	100±0.00	99.089 ± 0.14^{b}	98.610 ± 0.19^{b}	98.373 ± 0.18^{cd}	98.431 ± 0.19^{bc}
DCM: MethanolicLeaf extracts	Turpentine + 50 mg/kg	100±0.00	99.018 ± 0.12^{b}	98.626 ± 0.09^{b}	99.098 ± 0.09^{b}	99.554 ± 0.12^{a}
	Turpentine + 100 mg/kg	100±0.00	99.071 ± 0.04^{b}	98.544 ± 0.06^{b}	98.549 ± 0.06^{c}	98.808 ± 0.07^{b}
	Turpentine + 150 mg/kg	100±0.00	98.734 ± 0.23^{b}	98.239 ± 0.18^{b}	97.950 ± 0.18^{d}	97.892 ± 0.20^{c}

Note: Values are expressed as Mean ± SEM for five animals per group. Statistical comparisons were made within a column, and values with the same superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). Turpentine =20%; DMSO = 10%

Table 4. Effects of intraperitoneal administration of stem bark extracts of DCM-methanolic *X. americana* onturpentine-induced pyrexia in rats

Group	Treatment	Percent change in rectal temperature (°C) after drug administration				
		0h	1h	2h	3h	4h
Normal control	DMSO	100±0.00	99.973 ± 0.09^{ab}	99.995 ± 0.01^{a}	100.02 ± 0.09^{a}	100.01 ± 0.09^{a}
Negative control	Turpentine + DMSO	100±0.00	100.77 ± 0.09^{a}	100.82 ± 0.06^{a}	100.64 ± 0.11^{a}	100.48 ± 0.13^{a}
Positive control	Turpentine + DMSO + Aspirin	100±0.00	99.261 ± 0.08^{bc}	98.865 ± 0.09^{b}	98.329 ± 0.16^{b}	98.575 ± 0.09^{b}
DCM: Methanolic stem bark extracts	Turpentine + 50 mg/kg	100±0.00	99.088 ± 0.39^{c}	98.510± 0.47^{b}	98.562 ± 0.45^{b}	98.721 ± 0.49^{b}
	Turpentine + 100 mg/kg	100±0.00	99.288 ± 0.09^{bc}	98.459 ± 0.12^{b}	98.502 ± 0.14^{b}	98.723 ± 0.09^{b}
	Turpentine + 150 mg/kg	100±0.00	99.118 ± 0.15^{c}	98.131 ± 0.08^{b}	97.869 ± 0.19^{b}	97.984 ± 0.19^{b}

Note: Values are expressed as Mean ± SEM for five animals per group. According to a one-way ANOVA with Tukey's post hoc test, values with the same superscript are not significantly different within a column (p>0.05). Turpentine =20%; DMSO = 10%; Aspirin = 100 mg/kg

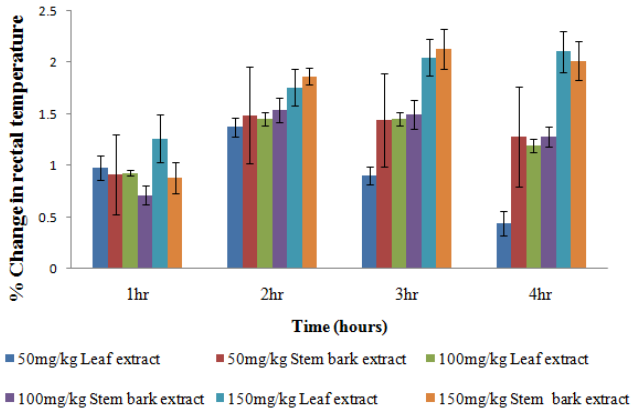

Figure 3. Comparison of change in rectal temperature of DCM-Methanolic leaf and stem bark extracts of *X. americana* on turpentine-induced pyrexia in rats

In the second hour after treatment, *X. americana* leaf extract DCM-methanol reduced paw circumference by 8.47%, 11.62%, and 7.85% at all doses (50, 100, and 150 mg/kg body weight), respectively. At this time, the anti-inflammatory activities of *X. americana* DCM methanolic leaf extract at the three dose levels were significantly different compared to the normal and negative control groups ($p<0.05$; Table 5). However, within this hour, the anti-inflammatory activity of the extract at doses of 50 and 150 mg/kg body weight was comparable to that of the positive control ($p>0.05$; Table 5). In the third hour after treatments, *X. americana*'s DCM methanolic leaf extract at doses of 50,100 and 150 mg/kg body weight reduced the paw circumference of the mice by 14.54%, 15.04%, and 16.90% (Figure 4). However, the anti-inflammatory activities of the DCM methanolic leaf extract of *X. americana* at all three dose levels differed significantly from the normal and negative control groups ($p<0.05$; Table 4), they were comparable to diclofenac ($p>0.05$, Table 5).

In the fourth hour of the trial period, *X. americana* at doses of 50, 100, and 150 mg/kg body weight reduced paw circumference in mice by 2.61%, 15.94%, and 9.01%, respectively (Figure 4). The activity of the extract at the three doses was significantly different compared to the normal and negative control groups ($p<0.05$; Table 4), but there was no significant difference between the positive control group and the dose of 100 mg/kg body weight ($p>0.05$, Table 5). Similarly, the DCM-methanolic stem bark extract of *X. americana* showed anti-inflammatory activity against carrageenan-induced inflammation in mice (Table 6; Figure 5). The reduction in leg circumference indicated this.

In the first hour after treatment, *X. americana* stem bark extract at the three dose levels of 50, 100 and 150 mg/kg body weight reduced the circumference of the inflamed hind limb by 5.848%, 9.174%, and 10.27% (Figure 5; Table 6). The anti-inflammatory activity of the extract at the three doses showed no significant difference compared to diclofenac (reference drug) ($p>0.05$; Table 6). However, the anti-inflammatory activity of the extract at the three doses of 50, 100, and 150 mg/kg body weight was significantly different compared to the negative control ($p<0.05$; Table 6).

During the second hour after treatment, *X. americana* extract at doses of 50, 100, and 150 mg/kg body weight reduced inflamed paw circumference in mice by 13.72%, 19 .17%, and 20.33%, respectively (Figure 5; Table 6). The anti-inflammatory effects of *X. americana* at the three doses were not significantly different and were comparable to diclofenac ($p>0.05$; Table 6). However, the anti-inflammatory activity of the extracts at the three doses (50, 100, and 150 mg/kg body weight) was significantly different compared to the normal and negative control groups ($p<0.05$; Table 6).

In the third hour after treatment, the extract at doses (50,100 and 150 mg/kg body weight) reduced the circumference of the inflamed hind limb in mice by 23.18%, 23.91%, and 25.97%, respectively (Figure 5). The anti-inflammatory activity of the extract at the three dose levels was not significantly different from each other nor the positive control ($p>0.05$; Table 6). However, the anti-inflammatory activity of the extract at the three dose levels was significantly different compared to the normal and negative control groups ($p<0.05$; Table 6; Figure 5).

Table 5. Effects of DCM-Methanolic leaf extract of *Ximenia americana* on carrageenan-inducedinflammation in mice

Group	Treatment	Percent change in paw circumference after drug administration				
		0h	1h	2h	3h	4h
Normal control	DMSO	100±0.00	99.997 ± 0.16^{a}	100.01 ± 0.15^{a}	99.921 ± 0.18^{a}	100.10 ± 0.10^{a}
Negative control	Carrageenan + DMSO	100±0.00	100.46 ± 0.18^{a}	103.46 ± 1.73^{a}	103.74 ± 1.73^{a}	103.79 ± 1.65^{a}
Positive control	Carrageenan + DMSO + Diclofenac	100±0.00	98.684 ± 0.40^{ab}	94.986 ± 0.67^{b}	82.34 ± 2.37^{b}	81.40 ± 1.46^{d}
DCM: Methanolic Leaf extracts	Carrageenan + 50 mg/kg	100±0.00	99.090 ± 0.22^{ab}	91.526 ± 0.98^{bc}	85.46 ± 1.71^{b}	97.39 ± 2.43^{ab}
	Carrageenan + 100 mg/kg	100±0.00	97.715 ± 0.58^{b}	88.38 ± 1.80^{c}	84.96 ± 1.85^{b}	84.06 ± 1.91^{cd}
	Carrageenan + 150 mg/kg	100±0.00	97.700 ± 0.87^{b}	92.149 ± 0.36^{bc}	83.10 ± 1.75^{b}	90.99 ± 2.24^{bc}

Note: Values are expressed as Mean ± SEM for five animals per group. Statistical comparisons were made within a column, and values with the same superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p>0.05$). Carragenan =1%; DMSO = 10%;Diclofenac = 15 mg/kg

Table 6. Effects of DCM-Methanolic stem bark extract of *Ximenia americana* on carrageenan-induced inflammationin mice

Group	Treatment	Percent change in paw circumference after drug administration				
		0h	1h	2h	3h	4h
Normal control	DMSO	100±0.00	100.00 ± 0.21^{b}	99.967 ± 0.06^{b}	100.06 ± 0.07^{b}	99.939 ± 0.06^{b}
Negative control	Carrageenan + DMSO	100±0.00	130.97 ± 3.85^{a}	133.45 ± 3.32^{a}	133.97 ± 3.44^{a}	134.34 ± 3.40^{a}
Positive control	Carrageenan + DMSO + Diclofenac	100±0.00	90.020 ± 0.43^{c}	79.915 ± 0.66^{c}	73.06 ± 1.97^{c}	70.40 ± 1.70^{c}
DCM: Methanolic stem bark extracts	Carrageenan + 50 mg/kg	100±0.00	94.160 ± 1.82^{bc}	86.28± 2.29^{c}	76.82 ± 1.40^{c}	91.38 ± 2.91^{b}
	Carrageenan + 100 mg/kg	100±0.00	90.826 ± 0.90^{c}	80.83± 1.83^{c}	76.09 ± 1.19^{c}	72.27 ± 1.80^{c}
	Carrageenan + 150 mg/kg	100±0.00	89.731 ± 0.68^{c}	79.67 ±1.60^{c}	74.03 ± 1.55^{c}	71.004 ± 0.94^{c}

Note: Values are expressed as Mean ± SEM for five animals per group. Statistical comparisons were made within a column, and values with the same superscript were not significantly different by one-way ANOVA followed by Tukey's post hoc test ($p > 0.05$). Carrageenan=1%; DMSO = 10%; Diclofenac = 15 mg/kg

In the fourth hour after treatment, the extract at doses of 50 mg/kg, 100 mg/kg, and 150 mg/kg body weight, as well as diclofenac, reduced the circumference of the inflamed hind leg by 8.62%, 27.73%, 29.00%, and 29.60%, respectively. (Figure 5; Table 6). Extracts at the dose level of 100 and 150 mg/kg body weight were comparable to each other and the positive control group (p>0.05; Table 6). However, the anti-inflammatory activity of the extract at a dose of 50 mg/kg body weight was significantly different from that of the positive control group (p<0.05; Table 6). The highest anti-inflammatory activity was observed at a dose of 150 mg/kg body weight, reducing the extent of the inflamed paw by 29%. However, the anti-inflammatory activity of the extract at a dose of 50 mg/kg body weight was significantly different from 100 mg/kg and 150 mg/kg body weight (p<0.05; Table 6) but comparable to the normal control group (p>0.05; Table 6; Figure 6).

In comparison, at a dose level of 50 mg/kg, there was a significant difference in the anti-inflammatory activity of the DCM-methanolic leaf and stem bark extracts of *X. americana* during the first and third hours of the trial period (p = 0.05 and 0.006 respectively). However, there was no significant difference at this dose level during the second and fourth hours (p= 0.08 and 0.157, respectively).

At the dose level of 100 mg/kg, there was a significant difference in the anti-inflammatory activity of the DCM-methanolic extracts of leaves and stem bark of *X. americana* in the 4-hour trial period (p= 0.001.0.022, respectively. 0.007 and 0.003).

At the dose level of 150 mg/kg, there was a significant difference in the anti-inflammatory activity of the DCM-methanolic extracts of leaves and stem bark of *X. americana* in the 4-hour trial period (p= 0.000,0.002, 0.006, and 0.000 respectively).

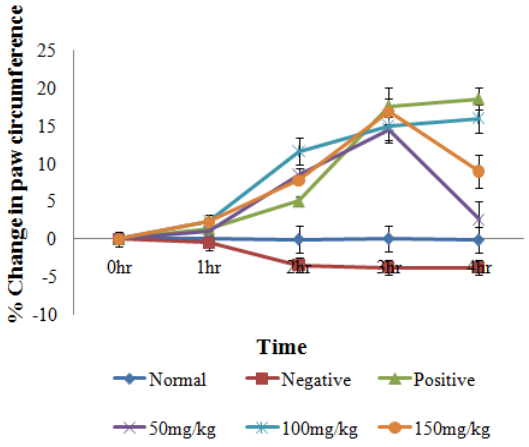

Figure 4. Anti-inflammatory effects of DCM-Methanolic leaf extract of *X. americana* on carrageenan-induced inflammation in the mice

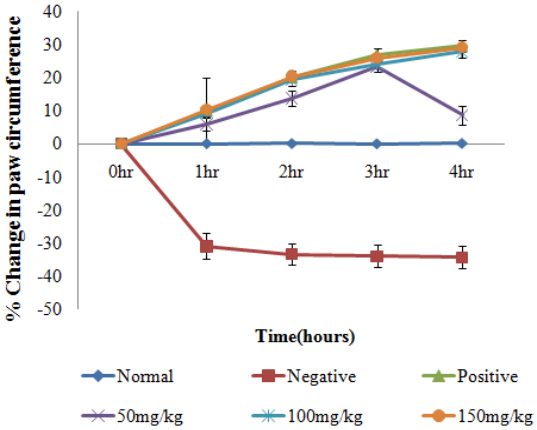

Figure 5. Anti-inflammatory effects of DCM-Methanolic stem bark extract of *Ximenia americana* on carrageenan-induced inflammation in mice

Phytochemical screening

Qualitative phytochemical screening of DCM-methanolic leaf and stem bark extracts from *X. americana* revealed the presence of alkaloids, cardiac glycosides, flavonoids, phenols, saponins, and terpenoids (Table 7). However, alkaloids and terpenoids were absent from *X. americana* leaf extracts, and steroids were absent from leaf and stem bark extracts.

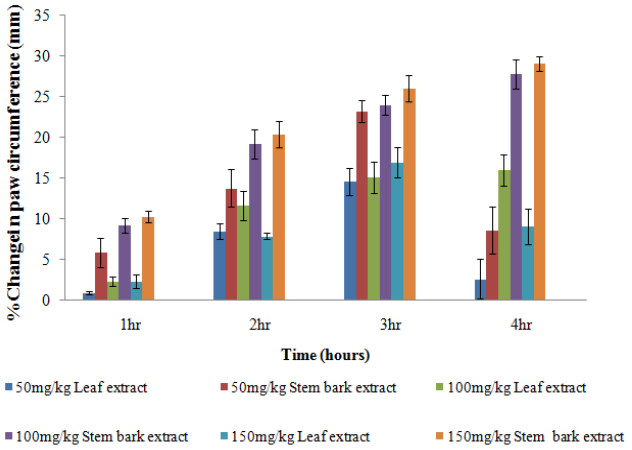

Figure 6. Comparison of Percent Reduction in Paw Circumference of DCM-Methanolic leaf and stem bark extract of *X. americana* on carrageenan-induced inflammation in mice

Table 7. Qualitative phytochemical composition of DCM-Methanolic leaf and stembark extracts of *X. americana*

Phytochemicals	*X. americana* leaf extract	*X. americana* stem barkextract
Alkaloids	-	+
Flavanoids	(+)	(+)
Steroids	-	-
Saponins	(+)	(+)
Cardiac glycosides	+	+
Phenolics	(+)	(+)
Terpenoids	-	+

Note: The (+) sign denotes present phytochemical; absent phytochemicals are denoted by the (-)sign, while + (trace) denotes slightly present phytochemical

Discussion

The present study aimed to evaluate the antipyretic and anti-inflammatory activities of DCM-methanolic leaf and stem bark extracts of *X. americana* in rat and mouse models. The antipyretic activity of the extracts was evaluated on turpentine-induced fever in male Wistar rats. Exogenous pyrogens such as steam distilled turpentine, lipopolysaccharides (LPS), brewer's yeast, muramyl dipeptide (MDP), and polyresin: polycytidylic acid (poly I: C) are used to induce fever in laboratory animals (Soszynski et al. 1991; Soszynski and Krajewska 2002). Turpentine is refined by distilling the resin from the pine. Intraperitoneal injection of distilled turpentine from the stem causes pyrexia as it improves prostaglandin synthesis (Zhu et al. 2011). Pyrexia caused by turpentine is rapid, and the tolerance of animals to turpentine is better than other exogenous pyrogens (Tung et al. 2006). This reason guided the choice of turpentine as an exogenous pyrogen for this study.

In this study of turpentine-induced pyrexia in rats (Tables 3 and 4), the significant antipyretic activity of the DCM-methanolic leaf and stem extracts of *X. americana* was observed. The results were consistent with other studies conducted on herbal extracts in laboratory animals. The aqueous extract of *X. americana* stem bark and its fractions showed antipyretic properties (Soro et al. 2015). In addition, Mwonjoria et al. (2011) showed that Solanum incanum root extract had an antipyretic effect on lipopolysaccharide-induced pyrexia in male Wistar rats.

Non-steroidal anti-inflammatory drugs inhibit the expression of cyclooxygenase 2 (COX-2), thereby inhibiting the biosynthesis of PGE2, leading to a reduction in elevated body temperature (Weissmann 1991). The antipyretic effect of many antipyretic drugs is achieved by inhibiting COX-2 and lowering PGE2 levels in the hypothalamus (Biren and Avinash 2010). COX-2 inhibitors are potent antipyretics and inhibit the conversion of arachidonic acid to PGE2 (Chandrasekharan et al. 2002). It was therefore believed that the DCM-Methanolic extracts of leaves and stem bark of *X. americana* reduced the rats' elevated rectal temperatures by inhibiting the COX-2 enzyme responsible for the production of PGE2 in the hypothalamus.

Dose levels of 50 mg/kg, 100 mg/kg, and 150 mg/kg were used in this study to evaluate the antipyretic activity of *X. americana* in rats with turpentine-induced pyretic. A similar assay was used by Gitahi (2015) to assess the antipyretic activity of dichloromethane: methanolic extracts of leaves and root bark of Carissa edulis in rats. Additionally, Akuodorl et al. (2013) used these dosages to evaluate the antipyretic effect of ethanolic extracts of Pseudocedrela kotschyi leaves in rats.

A dose-dependent effect was observed on the antipyretic activity of the methanolic extract of leaves and stem bark of X. American. The 150 mg/kg dose level produced the highest antipyretic activity (Figures 1 and 2; Tables 3 and 4). This dose-dependent result was similar to the work by Srivastava et al. (2013), who demonstrated the antipyretic activity of methanol extracts from Costus speciosus in experimental animals.

The antipyretic activity of the DCM-methanolic extracts of leaves and stem bark of *X. americana* were more efficient at the higher dose level of 150 mg/kg than at the dose level of 50 mg/kg and 100 mg/kg (Tables 3 and 2). Aspirin, the standard drug, showed an optimal antipyretic effect during the third hour (Figures 1 and 2; Tables 3 and 4), although its activity decreased after the third hour, which could be attributed to its metabolism and excretion. The maximum antipyretic effect of the DCM-methanolic extracts of leaves and stem bark of *X. americana* was observed in the third hour.

Passive diffusion of bioactive compounds across cell membranes into the abdominal cavity may contribute to this phenomenon (Hossain et al. 2011). The antipyretic activity of the leaf and stem bark extract of *X. americana* at a dose of 150 mg/kg body weight showed a greater effect than aspirin (Tables 3 and 4). Probably, the extracts inhibit prostaglandins better than the reference drug (aspirin) in these studies. The two parts of the plant did not differ significantly in their antipyretic activity. The presence of numerous phytochemical constituents in the DCM-methanolic extracts of leaves and stem bark of *X. americana* can be attributed to the antipyretic effects of the extracts. Phytochemical screening of the extracts revealed the presence of alkaloids, flavonoids, saponins, terpenoids,

cardiac glycosides, and phenols (Table 7). Several phytochemicals show antipyretic effects in experimental animals (Okokon and Nwafor 2010). Inhibition of prostaglandin synthesis has been reported by alkaloids (Niazi et al. 2010). Several phytochemicals have been hypothesized to explain the antipyretic effect of methanolic leaf and stem bark extracts in combination with DCM.

In the second hour after treatment, DCM-methanol *X. americana* leaf extract reduced leg circumference by 8.47%, 11.62%, and 7.85% at all doses (50, 100, and 150 mg/kg body weight, respectively). At this time, the anti-inflammatory activities of the methanolic leaf extract of *X. americana* DCM at the three dose levels were significantly different compared to the normal and negative control groups ($p<0.05$; Table 5). However, by this time, the anti-inflammatory activity of the extract at doses of 50 and 150 mg/kg body weight was comparable to that of the positive control ($p>0.05$; Table 5). In the third hour after treatments, *X. americana*'s DCM methanolic leaf extract at doses of 50,100 and 150 mg/kg body weight reduced the paw circumference of the mice by 14.54%, 15.04%, and 16%, 90% (Figure 4). However, the anti-inflammatory activities of the DCM methanolic leaf extract of *X. americana* at all three dose levels differed significantly from the normal and negative control groups ($p<0.05$; Table 4), they were comparable to diclofenac ($p>0.05$, Table 5).

Methanolic extracts of DCM leaves, and stem bark from *X. americana* produced significant anti-inflammatory effects on carrageenan-induced paw edema in Swiss albino mice (Tables 5 and 6). These results relate to the effects of other medicinal plants in animal models. For example, a study by Olabissi et al. (2011) showed that the aqueous ethanolic root bark extract of *X. americana* extract (10 and 100 mg/kg body weight) significantly inhibited carrageenin-induced swelling of mouse paws, with the intensity of the anti-edema effect of the aqueous extract being proportional to the doses tested. Similar work by Zakaria et al. (2007) using aqueous extract of Bauhinia purpurea leaves in animal models showed a significant anti-inflammatory effect on carrageenan-induced inflammation. Similarly, the work of Shashank et al. (2013) showed a potent anti-inflammatory effect of ethanolic and aqueous extracts of Kalanchoe pinnata on carrageenan-induced edema in rats. Non-steroidal drugs such as diclofenac, ibuprofen, indomethacin, and others are commonly prescribed in the treatment of inflammation (Fiorucci et al. 2000). These drugs work by blocking the enzyme cyclooxygenase (COX) to inhibit; the prostaglandin biosynthesis (Blandizzi et al. 2009). Therefore, the anti-inflammatory activities of leaf and stem bark extracts of *X. americana* to control inflammation can be attributed to the inhibition of prostaglandin synthesis.

The anti-inflammatory effect of DCM methanolic extracts of *X. americana* at all doses (50, 100, and 150 mg/kg body weight) over 3 hours in carrageenan-induced inflammation was comparable to that of the positive control group. Previous studies with plants like *Maytenus obscura* (A.Rich.) Cufod., *Caesalpinia volkensii* Harms (Mwangi et al. 2015), and *Solanum trilobatum* L. (Pandurangan et al. 2010) showed a similar effect in this model. Results demonstrate that late-phase extracts act dose-dependently involving arachidonic acid metabolites, as they are associated with neutrophil mobilization-dependent edema. This dose-dependent trend can be attributed to the diffusion of bioactive principles through the cell membrane into the peritoneal cavity in a passive manner.

On the dose levels of 50,100 and 150 mg/kg body weight were used to test the anti-inflammatory activity of the extracts in mice. Similar studies conducted by Vijay and Vijayvergia (2010), Mwonjoria et al. (2011), and Mwangi et al. (2015) used similar dose ranges in their studies when evaluating the anti-inflammatory effects of medicinal plants in animal models.

The *X. americana* DCM-Methanolic leaf and stem extracts exhibited similar anti-inflammatory activities to diclofenac. American leaf extracts reduced leg edema by 0.91%-16.90%, while stem bark extracts reduced it from 5.84% to 28.99%. Diclofenac reduced paw edema from 1.32% to 29.6%. Therefore, the extracts can be considered an effective anti-inflammatory agent similar to the reference medicinal product (diclofenac). On the anti-inflammatory activity, those two parts of the plant did not differ significantly. The anti-inflammatory activity of DCM-methanolic leaf and stem bark extracts of *X. americana* can be attributed to the presence of phytochemicals. Phytochemical screening revealed the presence of flavonoids, saponins, cardiac glycosides, and phenols in the leaf extract. In contrast, the stem bark extract found alkaloids, flavonoids, saponins, cardiac glycosides, phenols, and terpenoids (Table 7). Bhaskar and Balakrishnan (2009) report that these phytochemicals exert anti-inflammatory activity in animal models.

In this study, methanolic extracts of DCM leaves and stem bark from *X. americana* were shown to be antipyretic and anti-inflammatory. *X. americana* extract has powerful antipyretic and anti-inflammatory properties, as evidenced by the significant reduction in rectal temperature and paw circumference compared to the reference drugs. Thus, DCM-methanolic extracts of the leaves and stem bark of *X. americana* can treat fever and inflammation and provide an effective alternative to synthetic drugs. Furthermore, it was found that DCM-methanolic extracts of the leaves and stem bark of *X. americana* contain phytochemicals class associated with antipyretic and anti-inflammatory effects. Accordingly, the study supports the traditional use of *X. americana* to treat fevers and inflammations, rejecting the null hypothesis.

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